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*Neurobiological, neurobehavioural and  
metabolic correlates of  
Alzheimer's Disease in mouse models*

*Thesis for the Doctor of Philosophy Degree of*

*Alex Harper*

*University of London,  
Wolfson Institute for Biomedical Research  
Supervisor: Professor W. Richardson*

*Sponsored by GlaxoSmithKline  
Supervisor: Dr. C. Dingwall*

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## Publications resulting from this work

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### Conference Proceedings

- Alex Harper, Steve Harrison, Evelyn Grau, Tina Hamilton, Graham Duddy, Colin Dingwall. *Characterisation of  $\beta$ -Secretase (Asp2) overexpressing mice using a novel gene expression strategy*. (2001) Poster at the Society for Neuroscience meeting in San Diego.
- Alex Harper, Pippa Pugh, Panida Hemmati, Claire S Shilliam, Zoe A Hughes, Lee A. Dawson, Colin Dingwall. *Unexpected phenotype in BACE1 mice*. (2002) Poster at the Society for Neuroscience meeting in Orlando.
- Alex Harper, Alastair Morrison, Angela Austin, Sue Barber, Leanne Quinn, Evelyn Grau, Colin Dingwall. *BACE 1 & 2 double knockout mice and other worries*. (2003) Poster at the Society for Neuroscience meeting in New Orleans.
- Alex Harper, Haydn Prosser, Alastair Morrison, Angela Austin, Helen Chapman, Sue Barber, Leanne Quinn, Evelyn Grau, Colin Dingwall. *Anxiety in BACE1 & 2 double knockout mice*. (2004) Poster at the British Association for Psychopharmacology Summer meeting in Harrogate.
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## Abstract

Beta-site APP Cleaving Enzyme 1 (BACE1) is a key enzyme in the generation of  $\beta$ -amyloid, the major component of senile plaques in the brains of Alzheimer's Disease patients. Transgenic mice carrying human BACE1 cDNA and knockout mice with endogenous murine BACE1 removed were tested for behavioural, neurochemical and physiological changes with respect to appropriate wild type control mice. BACE1 transgenic mice exhibited a bold, exploratory behaviour, showed elevated 5-HT turnover and had unimpaired cognition. BACE1 knockout mice showed a contrasting behaviour, being timid and less exploratory with sex-dependent memory impairment as measured in the fear conditioning test. Unexpectedly these mouse lines did show alterations in their ability to put on weight suggesting an intriguing link between BACE1 and fat metabolism. Despite these clear differences both mouse lines were viable and fertile with no changes in morbidity.

The behaviour of a mouse line with both BACE1 and BACE2, a homologue of BACE1, endogenous genes knocked out was also investigated. The added deletion of endogenous BACE2 had no additional effect beyond that seen in BACE1 knockout mice. Compound transgenic mice carrying human BACE1 and Swedish mutant Amyloid Precursor Protein (APP<sub>KM670/671NL</sub>) transgenes or human mutant Presenilin 1 (PS1<sub>M146V</sub>) and APP<sub>KM670/671NL</sub> transgenes were tested for cognitive deficits. In combination with APP<sub>KM670/671NL</sub>, the presence of the BACE1 transgene had no effect on cognition but the presence of the PS1<sub>M146V</sub> transgene did result in impaired learning and memory.

The results presented in this thesis suggest an unexpected role for BACE1 in neurotransmission and fat metabolism, perhaps through changes in APP processing and  $\beta$ -amyloid levels although these effects may be unrelated to the role of BACE1 as  $\beta$ -secretase. As BACE1 inhibition is a target for Alzheimer's Disease modification, the potential effect of BACE1 therapeutic agents on anxiety and cognition should be considered.

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# *Chapter 1 - Introduction*

## 1.1 *Historical Perspective*

Alois Alzheimer first described the disease that was subsequently named after him in 1906 (1), publishing the description in 1907 (2;3). Born in 1864 in the small Bavarian town of Marktbreit, Alzheimer studied at the universities of Aschaffenburg, Tübingen, Berlin and Würzburg and in 1887 was granted his medical degree, defending his doctoral thesis on the wax producing glands of the ear in the same year. Initially he worked in Frankfurt am Main at the Städtische Irrenanstalt (mental asylum) where he became interested in neuropathology and met the neurologist, Franz Nissl. Alzheimer and Nissl worked together on the pathology of the nervous system, in particular the cerebral cortex. Their work was published in 6 volumes between 1906 and 1918 collectively entitled 'Histologische und Histopathologische Arbeiten über die Grosshirnrinde' (Histologic and Histopathologic Studies of the Cerebral Cortex).

In 1895, aged 31, Alzheimer became director of the Irrenanstalt and for 7

years continued to work on a wide range of subjects. In 1902 he was offered a post in Heidelberg University Psychiatric Clinic working for 'The Linnaeus of Psychiatry' Emil Kraepelin. Nissl had already moved to Heidelberg and so the two worked together again. In 1903 Alzheimer moved with Kraepelin to Munich university psychiatric clinic and in 1908 he joined the staff of the Psychiatric Institute as Ausserordentlicher (associate) professor and director of the clinic's anatomical laboratory. The laboratory soon gained an international reputation and became a meeting place for several important researchers, among

### **Alois Alzheimer**

them Ugo Cerletti, Hans Gerhardt Creutzfeldt, Alfons Maria Jakob, Fritz H. Lewy, F. Lotmar and Gaetano Perusini.

Almost one century before Alzheimer's work, in 1817, James Parkinson published 'An Essay on the Shaking Palsy' and stated that he believed cognitive decline was not part of Parkinsonism (4) thus establishing a differentiation between mental and physical affliction. In the early 1900s many researchers were working in the field of psychiatry and there was little distinction between the different diagnoses. The ability of researchers to differentiate between types of mental affliction was made easier with improvements made in histology and to the high powered achromatic lenses used in microscopy (5). Post mortem tissue sections from patients were examined for clues to the cause of the illness. This was the type of work that Alzheimer carried out and which proved so useful in finding pathological differences as the basis of disease symptoms.

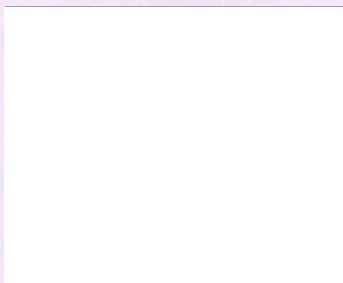
It was while working in Munich university that Alzheimer gave a presentation at a meeting of the South-West German Society of Alienists in November 1906 entitled "Eine Eigenartige Erkrankung der Hirnrinde" (A Peculiar Disease of the Cerebral Cortex) (1). The presentation described the clinical and neuropathological features of a woman aged 51 years who had died in the Munich mental asylum. The woman, named only as August D, began having various disparate symptoms 5 years previously. Initially she had fits of jealousy towards her husband and subsequently suffered from memory loss, hallucinations and became unable to care for herself while rejecting offers of help. Eventually her family was no longer able to cope and she was admitted to hospital. On hospitalisation her symptoms included disorientation, impaired memory, dyscalculia and dyslexia.

At the autopsy of August D, Alzheimer described three key observations previously unseen together. The cerebral cortex was thinner than normal; there were senile plaques and

#### **August D.**



neurofibrillary tangles which were positive for Bodian's silver stain. The presence of these structures together was novel and defined the new disease.



**Amyloid Plaques**



**Neurofibrillary Tangles**

The word amyloid is a derivative of amylum and comes from the Greek amulon (meaning fine meal or starch). The term amyloid refers to the atypical staining properties of autopsy specimens with iodine sulphuric acid as described by Virchow (6). Subsequently Alzheimer used this terminology to describe the plaque deposits he saw in the brain of August D.

Alzheimer made a preliminary report of his findings in 1906 and Perusini published 4 more cases in 1910 emphasising the differences between this condition and senility. Alzheimer published a full description of work on the disease to bear his name in 1911 which included a description of a second patient, Johann F (7;8). In this paper Alzheimer made reference to Kraepelin's 8<sup>th</sup> edition of his textbook on psychiatry (9) in which Kraepelin had described various aspects of specific types of senile dementia and had called them after Alzheimer. Thus the peculiar combination of altered behaviour, amyloid plaques and neurofibrillary tangles were classed together as a specific new illness called Alzheimer's Disease.

Historically the term dementia referred to any number of ailments where individuals were seen to be not in control of their faculties. Gradually, around the time Alzheimer was carrying out his studies, a distinction came to be made between mental defects that were pre-existing – termed amentia – and those that developed over a period of time – termed dementia (10). In his paper on the history of the use of the term dementia published in the American Journal of Insanity, Blumer outlines the thinking of the day and makes extensive reference to the 7<sup>th</sup> edition of Kraepelin's book on psychiatry (11). It was clear that while attempts were being made to classify different forms of dementia by behaviour, it took the scientific investigative research of Alzheimer and his peers to identify accurately the

histological lesions that came to be recognised as the hallmarks of AD. Subsequent classification of different types of dementia using histology in combination with behaviour enabled a more rigorous diagnosis of the disease to be performed, albeit post mortem.

## ***1.2 Alzheimer's Disease in the Post-genomic Era***

During the following 8 decades AD research focussed mainly on genetics and the discrimination of the disease from other dementias, leading to the realisation that the symptoms of AD, including memory loss, were not part of aging but were actually disease related. In the early part of the last century a number of papers showed that dementia had a familial component (12-14). AD patients were shown to have amyloid plaques containing degenerating nerve endings and their plaque count far exceeded that found in normal aging (15;16). Terry and Davies (17) indicated that the presenile form of dementia (with onset before age 65) is identical to the most common form of senile dementia, recommending the condition be designated as senile dementia of the Alzheimer type (SDAT). Application of the modern biological technologies used in molecular and cell biology allowed a more detailed understanding of AD biology. Masters and colleagues eliminated the probability of a viral component of AD when they published a study of 52 families with AD affected members comparing them to familial Creutzfeldt-Jakob disease (18). A key breakthrough was the identification of a novel amyloid protein in the plaques of AD patients, called beta protein (19). Analysis of the beta protein cDNA suggested it was derived from a larger precursor protein with distribution in a large number of tissues (20). Key papers followed including those which described the cloning of the APP gene (21) and the molecular genetic approach to finding genes associated with the disease (22). With the improved antibody reagents available a clearer understanding of proteins involved in the formation of neurofibrillary tangle was possible (23) and the microtubule associated protein Tau (MAPT or simply  $\tau$ ) was identified as a key component of the Neurofibrillary tangles (24;25). However a unifying hypothesis of how the different proteins interacted to produce the disease was not published until 1991 with the Amyloid Cascade Hypothesis (26;27). Even now this hypothesis is not wholly accepted by all with various researchers falling into the 'Baptist' or 'Tauist' camps. Each camp has differing opinions as to the correct aetiology of the disease

though some common themes are present and one unified hypothesis may be possible (28;29). Strong evidence in support of the amyloid cascade hypothesis was presented when, in a triple transgenic mouse model expressing 3 mutant transgenes (APP<sub>SWE</sub>, PS1<sub>M146V</sub> and  $\tau$ <sub>P301L</sub>) was found to develop plaque pathology prior to tangle pathology (30).

Disease	Etiology	Regions most affected	Characteristic pathology	Disease proteins deposited
Huntington's disease	Huntingtin (dominant)	Striatum, other basal ganglia, cortex, other regions	Intranuclear inclusions and cytoplasmic aggregates	Huntingtin with polyglutamine expansion
Other polyglutamine diseases (DRPLA, SCA1–3, etc., SBMA)	Atrophin-1, ataxin-1–3, etc.; androgen receptor (AR) (dominant)	Basal ganglia, brain stem cerebellum, and spinal cord	Intranuclear inclusions	Atrophin-1, ataxins or AR
Alzheimer's disease (AD)	Sporadic (ApoE risk factor)	Cortex, hippocampus, basal forebrain, brain stem	Neuritic plaques and neurofibrillary tangles	A $\beta$ peptide (from APP) and hyper-phosphorylated tau
	Amyloid precursor protein (APP) (dominant)	Same as sporadic	Same as sporadic	Same as sporadic
	Presenilin 1, 2 (dominant)	Same as sporadic	Same as sporadic	Same as sporadic
Fronto-temporal dementia with Parkinsonism	Tau mutations (dominant)	Frontal and temporal cortex, hippocampus	Pick bodies	Hyper-phosphorylated tau protein
Parkinson's disease (PD)	Sporadic	Substantia nigra, cortex, locus ceruleus, raphe, etc.	Lewy bodies and Lewy neurites	$\alpha$ -Synuclein
	$\alpha$ -Synuclein (dominant)	Similar to sporadic, but more widespread	Similar to sporadic	$\alpha$ -Synuclein
	Parkin (also DJ-1, PINK1) recessive (some dominant)	Substantia nigra	Lewy bodies absent (or much less frequent)	$\alpha$ -Synuclein (when present)
Amyotrophic lateral sclerosis (ALS)	Sporadic	Spinal motor neurons and motor cortex	Bonina bodies and axonal spheroids	Unknown (neurofilaments)
	Superoxide dismutase-1 (dominant)	Same as sporadic	Same	Unknown
Prion diseases (kuru, CJD, GSS disease, fatal familial insomnia, new variant CJD)	Sporadic, genetic and infectious	Cortex, thalamus, brain stem, cerebellum, other areas	Spongiform degeneration, amyloid, other aggregates	Prion protein

ApoE, apolipoprotein E; APP, amyloid precursor protein; CJD, Creutzfeldt–Jakob disease; DRPLA, dentato-rubral and pallido-Luysian atrophy; GSS, Gerstmann–Straussler–Scheinker; SBMA, spinal and bulbar muscular atrophy; SCA, spino-cerebellar ataxia.

**Table 1.1 Diagnostic characteristics of a range of neurodegenerative diseases.**

The term Neurodegeneration refers to the death of neurons and when this occurs in neurons of the Central Nervous System it leads to a progressive shrinking of the brain. This condition is known as dementia and, as many of the patients in this condition are elderly, the term senile dementia has been used to describe the chronic neurodegenerative process (31). The causes of dementia are many but a relatively small range of disease syndromes account for the majority of neurodegenerative processes in the brain. Each disease has numerous sub-divisions and classifications but they can broadly be split according to the type of lesion found in the brain. These diseases are; Alzheimer's Disease, which is characterised by neuronal plaque with tangles; Parkinson's Disease, which is characterised by Lewy bodies and Frontal Temporal Dementia which is characterised by tangles alone. These diseases do have overlapping pathology and symptoms and as more is discovered about the biology of these diseases more common features may be found. Table 1 lists various neurodegenerative diseases and the characteristics which define each disease (32). AD along with Parkinson's Disease (PD) and Amyotrophic Lateral Sclerosis (ALS) are unusual in as much that they have sporadic forms where the disease occurs in the absence of genetic linkage and familial forms where mutations in specific proteins pre-dispose an individual to the disease. This genetic component to the diseases has proved very valuable for researchers attempting to model the disease.

AD is the most prevalent of all adult onset neurodegenerative disorders and is characterised by a progressive memory loss that may also be associated with other neuropsychiatric and behavioural impairments. The principle neuropathological features of this disease are overt neuronal and synaptic loss, extra-cellular accumulation of amyloid plaques composed primarily of  $\beta$ -amyloid peptide and intra-cellular neurofibrillary tangles composed of hyperphosphorylated  $\tau$  (33). These defining hallmarks of AD neuropathology have been the focus of intense research over the past two decades and have led to the identification a key enzyme involved in  $\beta$ -amyloid production, as well as to the identification of a variety of kinases that can potentially hyperphosphorylate  $\tau$  *in vivo*. While the Amyloid Precursor Protein (APP) promoter may be important in the progression of AD pathogenesis (34) a number of mutations of the APP protein have been identified that are associated with the prevalence of AD (see table below).

Mutation	Phenotype	Age of Onset	References
Glu665Asp	AD, but may not be pathogenic *	Approx 86	(35)
Lys/Met670/Asn/Leu (Swedish)	AD	52 (44-59)	(36)
Ala673Thr	Normal	N/A	(37)
His677Arg	AD	55 (55-56)	(38)
D678N	FAD	60	(39)
Ala692Gly (Flemish)	Large dense core plaques linked to CAA	40-60 but variable	(40-43)
Glu693Gly (Arctic)	AD, but may not be pathogenic * ↓ plasma Aβ40 & 42, high protofibril levels	58? N/A	(44) (45)
Glu693Gln (Dutch)	HCHWA-D (a stroke syndrome)	Typically ~50 but variable	(46)
Glu693Lys (Italian)	Cerebral Haemorrhage	?	(47)
Asp694Asn (Iowa)	AD or Cerebral Haemorrhage	69	(48)
Ala713Thr	AD, but may not be pathogenic *	59	(49)
Ala713Val	Schizophrenia: probably not pathogenic *	?	(50)
Thr714Ile (Austrian)	Affects γ-secretase cleavage directly, 11X ↑ Aβ(42:40) ratio <i>in vitro</i> . In brain, abundant and predominant non-fibrillar pre-amyloid plaques made of N-truncated Aβ(42) in absence of Aβ(40).		(51)
Thr714Ala (Iranian)	AD	52 (40-60)	(52)
Val715Met (French)	AD	52 (40-60)	(53)
Val715Ala (German)	AD	47	(54;55)
Ile716Val (Florida)	AD	55	(56)
Val717Phe	AD	47 (42-52)	(57)
Val717Gly	AD	55 (45-62)	(58)
Val717Ile (London)	AD	55 (50-60)	(59)
Val717Leu (Indiana)	CSF tau and A-β consistent with AD; earlier onset than other 717 mutations	38	(60)
Leu723Pro (Australian)	AD	56 (45-60)	(61)

**Table 1.2 Pathogenic mutations in APP**

Adapted from <http://www.alzforum.org/>. \* Mutation found in AD patient but is rare and may be a normal polymorphism and not directly disease related.

The Amyloid Cascade Hypothesis places the β-amyloid protein central to AD pathology. The hypothesis states that either genetic or sporadic factors lead to an increase in the production of Aβ42 (one of the peptides derived from the action of two key secretase enzymes on APP, namely β- & γ-secretase) which aggregates and accumulates in the limbic and association cortices of AD patients. β-amyloid

oligomers exert subtle effects on synaptic plasticity and a gradual deposition of A $\beta$ 42 oligomers as diffuse plaques follow, stimulating microglial and astrocytic activation with altered neuronal ionic homeostasis and oxidative injury. Subsequent to  $\beta$ -amyloid production the hypothesis states that the other major feature of AD, neurofibrillary tangles (NFTs), are generated with altered kinase / phosphatase activity leading to hyperphosphorylated  $\tau$  and NFT tangle formation. The presence of plaques and tangles is thought to lead to widespread neuronal and synaptic dysfunction and some neuronal loss with neurotransmitter defects. These cellular changes manifest as the clinical symptoms of onset of dementia.

The amyloid cascade hypothesis has been updated from the original publication in 1991 to include new discoveries (33). A number of variations of this dogma in AD research have been published which among others include aluminium (62), inflammation (63), mitochondria (64) or cholesterol (65) as more or less important in the cascade. Others have challenged the hypothesis arguing that  $\beta$ -amyloid functions as a protective adaptation to AD (66) or that  $\tau$  is the central initiator of disease since neurofibrillary tangles alone can cause neurodegeneration (67). Interestingly a recent report has shown that persistence of NFTs in a mouse model of transient mutant  $\tau$  expression ( $\tau_{P301L}$ ) was insufficient to cause cognitive decline or neuronal death when the transgene expression was turned off, even though these features were seen when the transgene expression was switched on (68).

Prior to the formation of the amyloid cascade hypothesis, the cholinergic hypothesis of geriatric memory dysfunction was put forward in 1982 (69). The hypothesis states that the loss of cholinergic function in the CNS contributes significantly to the cognitive symptoms associated with AD and advanced age. The paper written by Bartus and colleagues generated considerable interest, ranking 4<sup>th</sup> among all neuroscience papers cited in the last 15 years. The hypothesis has proved to be of value as the only currently marketed treatments for AD do not modify the disease but use cholinesterase inhibitors to alleviate the symptoms. A more recent review of the cholinergic hypothesis describes the progress made with models and treatment strategies for neurodegenerative diseases (70). In his review Bartus points out that while disease modification based on the amyloid cascade hypothesis may yield a treatment in the future, the current symptomatic treatments are vital to continue treating those for whom disease modification will be of little use.



As already described AD can occur sporadically or shows a familial component. Four genes have been identified that account for approximately half of the genetic risk for inheriting AD. These genes are Presenilin 1 (PS1), Presenilin 2 (PS2), amyloid precursor protein (APP) and apolipoprotein E (ApoE) (71). Studies on the effects of mutations or polymorphisms in these genes have demonstrated that they alter the processing of APP and the sequelae outlined in the amyloid cascade hypothesis.

In addition to these genes, a number of others have been implicated in the disease progression of both sporadic and familial AD. A major component of AD pathology is the NFTs present in the brains of AD patients as well as a range of other related neurodegenerative disorders.  $\tau$  is the primary component of these tangle structures and several mutations have been found in this protein but there appears to be little correlation between the mutations and clinical presentation of the disease (72). Many other genes have now been discovered that have a variety of effects in the pathogenesis of AD. Genes that promote  $\beta$ -amyloid degradation such as Neprilysin (73) and Prolyl Isomerase (74) have been described as well as Receptor for Advanced Glycosylation Endproducts (RAGE) and LDL Receptor Protein (LRP) which regulate  $\beta$ -amyloid homeostasis in the CNS (75). The genetics of AD remains the subject of intense ongoing research with the role of genes directly involved in  $\beta$ -amyloid and  $\tau$  modulation as well as neuroinflammation and cell death still to be clearly defined and new genes to be discovered.

### ***1.3 Molecular Biology of Alzheimer's Disease***

The main constituent protein found in the cerebral plaques of AD patients was found to be an aggregated form of a novel 4 kDa protein named beta protein which was derived from a larger amyloid precursor protein (APP). At least three enzymes,  $\alpha$ -,  $\beta$ - and  $\gamma$ -secretase cleave APP.  $\alpha$ - plus  $\gamma$ -secretase cleavage leads to non-pathogenic processing of APP and the cleaved protein fragments are cleared from the brain.  $\beta$ - plus  $\gamma$ -secretase cleavage of APP generate the  $\beta$ -amyloid fragment which subsequently accumulates in the brains of AD patients to produce amyloid plaques that are one of the hallmarks of this disease. While  $\beta$ -amyloid is the core component of the amyloidogenic process contributing to AD, it is generated by all cells during normal metabolism (76).  $\beta$ -amyloid has toxic and neurotrophic (77)

properties and yet the role of this protein in peripheral tissues is not clear. Proposed functions for  $\beta$ -amyloid include a role as a sealant for the blood-brain barrier (78), as an essential synaptic protein (79) and serving as an anti-oxidant in the aging brain (80). In addition the  $\beta$ -amyloid channel hypothesis (81) has been proposed in which hydrophobic polymerised  $\beta$ -amyloid protein can infiltrate the lipid membrane bi-layer and form channels through which calcium ions can pass leading to the disruption of neuronal cells and mitochondria homeostasis.

AD is postulated to be a disease resulting from protein mis-folding (82) where the normal production and clearance of  $\beta$ -amyloid and  $\tau$  is somehow impaired and the proteins accumulate into respective plaques and tangles with associated neuronal cell damage. A number key proteins and structures in the pathogenesis of AD have been identified. These are discussed below along with current hypotheses for the disease progression.

### **1.3.1 Amyloid Precursor Protein**

APP is a trans-membrane protein with a large extra-cellular N-terminus and a small intra-cellular C-terminus (Figure 1.1). The genomic locus of APP is localised to chromosome 21q21 spanning 170 kb. The promoter region has been shown to have two blocks of regulatory sequences. One block extends from -600 to -460 bp and acts as a positive regulator. A second block of sequences extending from -450 to -150 bp acts in the same way as a negative regulator. A 38-mer synthetic oligonucleotide encompassing the region -489 to -452 of the APP promoter may also act as part of an enhancer-like element (83).

The APP gene comprises 19 exons which generate a protein of up to 770 amino acids. APP is expressed throughout the body with 3 main isoforms. These include APP695 (exons 1-6, 9-18, not 13a), APP751 (exons 1-7, 9-18, not 13a), APP770 (exons 1-18, not 13a). APP695 is the predominant neuronal isoform with APP751 most prominent throughout the rest of the body (84). They differ in that APP751 and APP770 contain exon 7, which encodes a serine protease inhibitor domain.  $\beta$ -amyloid is derived from that part of the protein encoded by parts of exons 16 and 17 (see below). Quantitative mRNA analyses of human brain samples showed that APP695 and APP751 expression levels were variable but were not changed in AD patients whereas APP770 was significantly elevated (85).



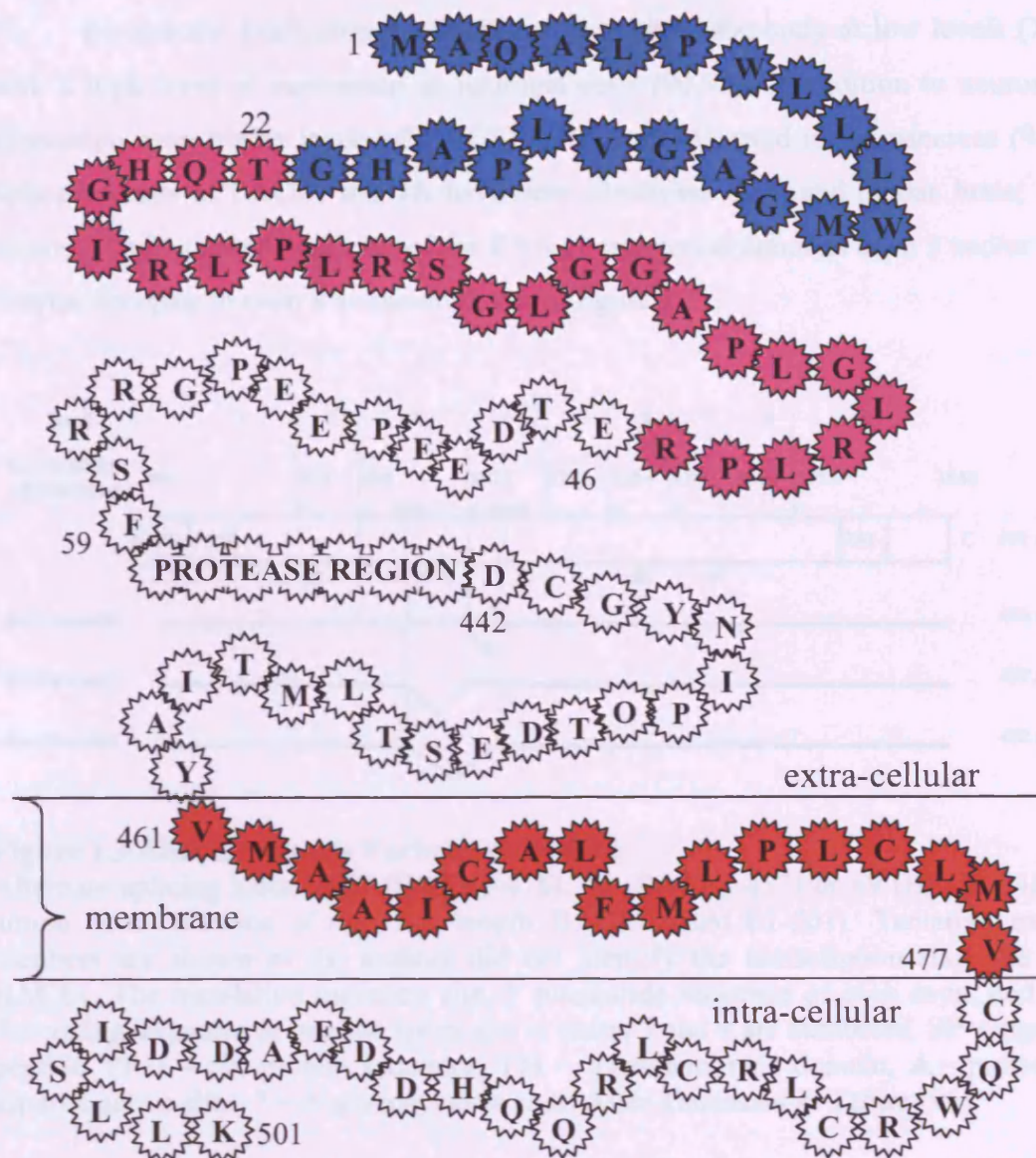
processing of APP to  $\beta$ -amyloid by secretases can occur in an axonal membrane compartment transported by kinesin-1 (87).

### 1.3.2 $\beta$ -secretase

$\beta$ -secretase was first cloned and published in 1999 by four independent groups (88-91). Each of these used different methods to identify the enzyme. Hussain *et al* (88) showed that point mutations in  $\beta$ -secretase (which they called Asp2) at both of its active sites (asp--ser/thr--gly sequence) resulted in a protein that could no longer process APP to  $\beta$ -amyloid. The publication from Sinha *et al* (89) described a protein fractionation approach to purify  $\beta$ -secretase (which they named p501). Several substrate analogue inhibitor variants of the APP sequence spanning the beta site, including transition-state analogues, were designed and used to pull out their candidate protease from human brain extracts, with a 300,000-fold enrichment. They described a membrane-bound enzyme activity that cleaved full-length APP at the  $\beta$ -secretase cleavage site, and found it to be the predominant beta-cleavage activity in human brain. Vasser *et al* (90) used an expression cloning strategy to clone a human trans-membrane aspartic protease with all the known characteristics of  $\beta$ -secretase and they named this protein BACE1 (Beta site APP Cleaving Enzyme 1). Yan *et al* (91) deduced that  $\beta$ -secretase has aspartyl – protease - like characteristics from pharmacological data. They demonstrated that solubilised  $\beta$ -secretase protein (which they called ASP2) cleaved a synthetic APP peptide substrate at the  $\beta$ -secretase site. Although  $\beta$ -secretase has been published using a variety of names including ASP2 and Memapsin 2, the name settled on is BACE1. Hereinafter the term BACE1 will be used for  $\beta$ -secretase.

BACE1 maps to chromosome region 11q23.2-q23.3 (92). BACE1 protein comprises 501 amino acids containing a 21-amino acid signal peptide followed by a 23-amino acid pro-protein domain and the sequence is shown diagrammatically in figure 1.2 overleaf. BACE1 is a type 1 trans-membrane protein with a single catalytic domain formed from two active sites on the luminal domain encompassing amino acids 93-95 [DTG] and 289-291 [DSG]. The luminal domain of the mature protein is followed by a trans-membrane domain (amino acids 461-477) and a short cytosolic C-terminal tail of 24 amino acids. On folding the protease is activated to allow the cleavage of APP at the  $\beta$ -secretase site.



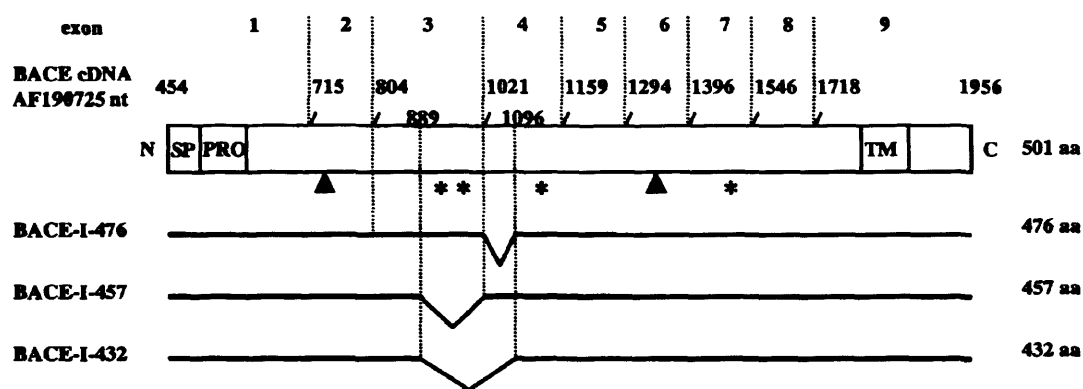


**Figure 1.2 Diagrammatical representation of BACE1 protein.**

The Pre- & Pro- BACE peptide sequences are shown in blue and pink respectively and the intra-membrane region in red. Adapted from the AD Forum Website: <http://www.alzforum.org/res/com/mut/app/diagram1.asp>

Northern blot analysis of human BACE1 mRNA in adult peripheral tissues and various sub-regions of the brain detected 3 transcripts of approximately 7.0, 4.4, and 2.6 kb. By *in situ* hybridization, expression of BACE mRNA in rat brain was observed at higher levels in neurons than in glia (90) supporting the idea that neurons are the primary source of the extra-cellular A $\beta$  deposited in amyloid plaques. Vassar and colleagues (90) ascribed the difference between the apparent and calculated molecular weight (approximately 70 and 51 kD, respectively) of the BACE protein to N-linked glycosylation. Immunostaining demonstrated intra-cellular localization of BACE1 to the Golgi and endosomes.

$\beta$ -secretase expression is distributed throughout the body at low levels (76) with a high level of expression in neuronal cells (90;93). In addition to neuronal expression even higher levels of BACE1 mRNA are detected in the pancreas (94). Splice variants of BACE1 mRNA have been identified in rat and human brain, all resulting from alternate splicing of the RNA at an internal donor in exon 3 and/or an internal acceptor in exon 4 as shown below in figure 1.3.



**Figure 1.3 Alternate Splice Variants of BACE1**

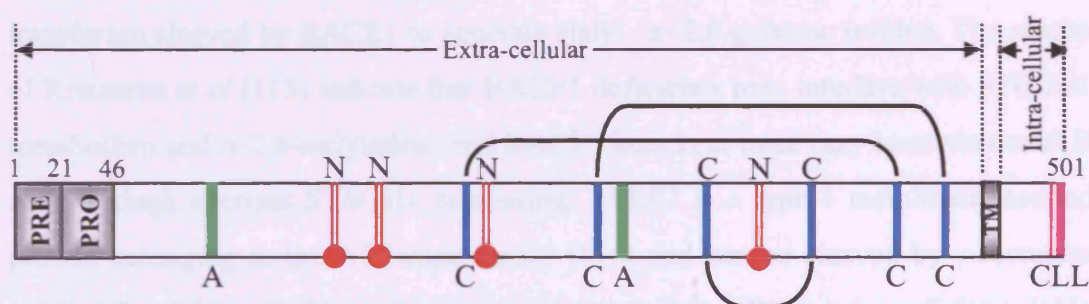
Alternate splicing leads to 25 (BACE1-476), 44 (BACE1-457) or 69 (BACE1-432) amino acid deletions of the full length BACE1 (BACE1-501). Tentative exon numbers are shown as the authors did not identify the transcription start site of BACE1. The translation initiation site, 5' nucleotide sequence of each exon, end of the coding sequence & internal splice site in exons 3 and 4 are numbered. SP = signal peptide, PRO = pro-protein sequence, TM = transmembrane domain, ▲ = protease aspartic active sites, \* = *N*-glycosylation sites. After Tanahashi & Tabira (95).

Pulse-chase experiments by the authors of figure 1.3 indicate that BACE1-476 and BACE1-457 are not processed in the cell Golgi. Characterisation of BACE1 RNA expression in neuronal tissue has shown that human frontal cortex has 3 times the level of BACE1 expression as human cerebellum and 4 times the level in rat frontal cortex. Neither of these latter regions develops  $\beta$ -amyloid plaques. Most human and rat brain regions express all 4 splice variants of BACE1, though BACE1-457 is absent from human cerebellum (96). These species specific differences in BACE1 transcripts may indicate differences in the evolutionary conservation of the gene.

BACE1 is an important target for therapeutic intervention in AD. Consequently the protein, represented diagrammatically in Figure 1.2, has been well characterised as part of target validation efforts by various different groups. BACE1



is a pepsin family member with unusual properties and thus represents a novel class of aspartyl proteases (97). The newly synthesised BACE1 propeptide is processed in the secretory pathway by furin or a furin-like propeptide convertase (98). Although APP cleavage by BACE1 has been reported to be in the endoplasmic reticulum and Golgi, endosomes are likely to be the major site for BACE1 activity owing to the acidic pH optimum of the enzyme (99). Micro-domains may exist within the cell where BACE1 may cleave APP at or near its pH optimum though these domains have yet to be found. BACE1 contains a cytoplasmic dileucine motif and the mutation of this dileucine to dialanine (100) increases the levels of BACE1 on the cell surface and decreases BACE1 internalisation. This is consistent with a role for the cytoplasmic tail of BACE1 in trafficking and localisation. He *et al* (101) showed that the Asp<sup>496</sup>, Leu<sup>499</sup> and Leu<sup>500</sup> residues in BACE1 were essential for successful binding to GGA (Golgi-localised  $\gamma$ -ear-containing ARF binding), a protein involved in the regulation of intra-cellular transport (102). In addition to data on cellular trafficking, all of the Reticulon family members, RTN1, RTN2, RTN3 and RTN4 (the last of which is also known as Nogo) have been shown to be binding partners for BACE1. Increase in the expression of any of these reticulon family members has been claimed to cause a reduction in  $\beta$ -amyloid production by blocking the access of BACE1 to APP (103).



**Figure 1.4 Key features of the BACE1 polypeptide**

The Pre-, Pro- and Trans-membrane regions are indicated as are the Extra- and Intra-cellular regions. A=Active sites, N=N-glycosylation sites, C=Cysteine residues with appropriate intramolecular disulphide linkages, L=Leucine. Adapted from (97).

The C-terminal cytoplasmic tail of BACE1 interacts with the copper chaperone for SOD1 (CCS) and this interaction remains stable enough for both of these proteins to be co-transported through axons. Additionally a peptide encompassing the BACE1 cytoplasmic domain binds a single Cu (I) atom with high

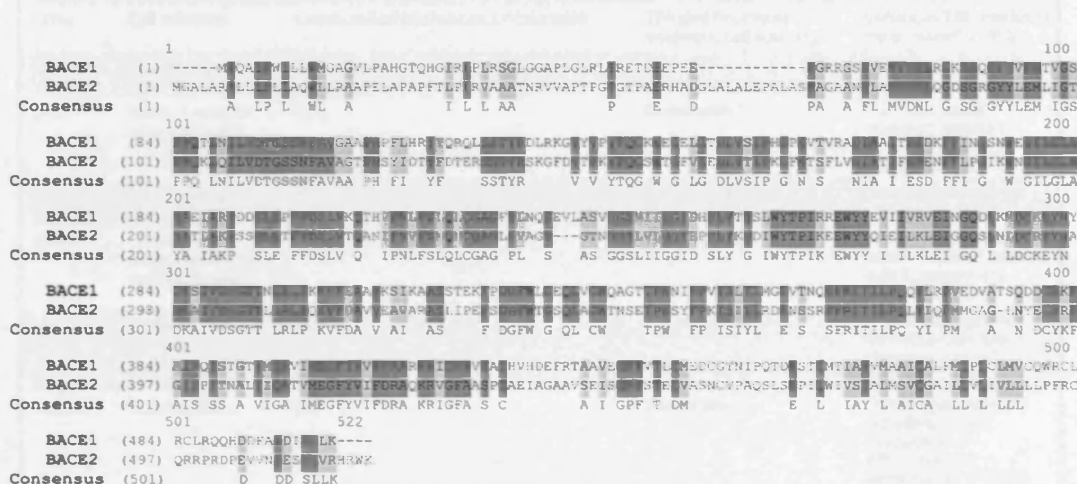
affinity. These data point towards the role BACE1 may play in copper metabolism and  $\beta$ -amyloid generation. These properties would also be evident in inactive BACE1 suggesting a non-proteolytic role for BACE1 linked to metal homeostasis (104).

The extra-cellular domain of BACE1 is shed, a process that has been shown to be controlled by a metalloproteinase. ADAM10 is a strong candidate for BACE1 sheddase (105). Recently two papers have suggested a mechanism for BACE1 activation whereby BACE1 dimerises to generate an active enzyme as long as it is still attached to the membrane whereas the monomeric soluble ectodomain has reduced aspartyl protease activity (106;107). To date no mutations have been found in BACE1 which are linked to AD incidence (108) although BACE1 polymorphisms play a role in AD (109).

While the primary function of BACE1 appears to be the processing of APP alternative roles for the protein may yet be discovered. Alternative substrates for BACE1 are P-selectin glycoprotein ligand-1 (110), the sialyl-transferase ST6Gal1 (111), Amyloid precursor like protein 2 (APLP2) (112), a voltage gated sodium channel  $\beta$ -subunit (VGSC $\beta$ ) (113) and the lipoprotein-related receptor protein (LRP) (114). P-selectin glycoprotein ligand-1 is expressed as a homodimer and mediates leukocyte adhesion to endothelial cells and is critically involved in the inflammatory response both in brain and in peripheral tissues. ST6Gal1 is a Golgi resident sialyl-transferase cleaved by BACE1 to generate sialyl-  $\alpha$ -2,6-galactose residue. The results of Kitazume *et al* (115) indicate that BACE1 deficiency may interfere with ST6Gal1 metabolism and  $\alpha$ -2,6-sialylation, and BACE1 knockout mice may have abnormal B cells through aberrant ST6Gal1 processing. APLP2 is a type 1 membrane-inserted protein belonging to the APP-superfamily (116) and can be cleaved by  $\gamma$ -secretase (117). Like APP, APLP2 can be processed to generate a large extra-cellular soluble ectodomain (118). APLP2 can not contribute to the  $\beta$ -amyloid peptide as it does not contain the  $\beta$ -amyloid sequence however APLP2 processing is modulated by BACE1 *in vivo* (119).

### 1.3.3 BACE2

BACE2 was cloned by a number of groups and the first papers were published around the same time as BACE1 (92;99;120-122). BACE2 has 52% homology to BACE1 and contains the same two DTGS & DSGT motifs characteristic of aspartyl proteases (123). Both enzymes are type 1 trans-membrane proteins with a short cytoplasmic tail, a single trans-membrane region and a large extra-cellular domain containing the active site (Figure 1.5). BACE2 has been shown to cleave APP at the  $\beta$ -secretase site as well as other sites within the  $\beta$ -amyloid fragment (124) though the enzyme cleaves at greater efficiency at 2 sites within the  $\beta$ -amyloid domain of APP (Phe +19 and Phe +20) near the  $\alpha$ -secretase cleavage site. BACE2 is thought to operate more as an  $\alpha$ -secretase enzyme with processing of APP by BACE2 leading to reduced  $\beta$ -amyloid production (125).



**Figure 1.5 Comparative amino acid sequences of BACE1 & BACE2**  
Dark shading indicates identical amino acid sequences and light shading represents similar sequences. Adapted from Sun *et al* (126).

BACE2 is distributed throughout the body with low mRNA levels detected in most human organs and higher expression in colon, kidney, pancreas, placenta, prostate, stomach and trachea. Interestingly BACE2 mRNA is found in low levels in the CNS (121). Both BACE1 and BACE2 have been reported to participate in the normal and abnormal processes in human muscle suggesting a broader function beyond that of a  $\beta$ -secretase (127).



### 1.3.4 $\gamma$ -secretase

$\gamma$ -secretase is a complex of proteins including Presenilin 1 (PS1) (128) in addition to Nicastrin, anterior pharynx (APH1) and presenilin enhancer 2 (PEN2) (129). When co-expressed in yeast, an organism that lacks endogenous  $\gamma$ -secretase activity, these four proteins were sufficient to reconstitute  $\gamma$ -secretase activity (130).

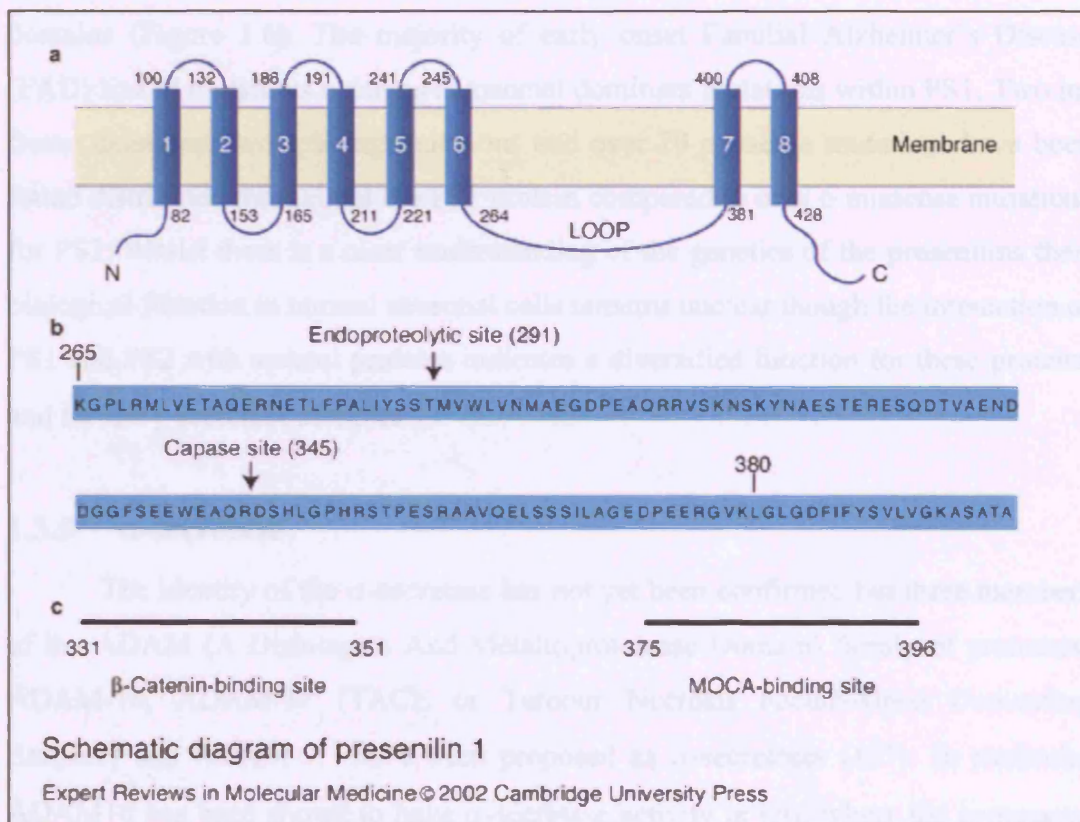
Substrate	Known role	$\gamma$ -Secretase-mediated cleavage sites*	Mechanism regulating ectodomain shedding/ $\gamma$ -secretase cleavage	Function of cleavage (FOC) or of released ICD (FOICD)
APP	Pathological role in Alzheimer's disease; unknown physiological role (transport/adhesion?)	<b>SNKGAIIGLMVGGVY</b> † <b>IA</b> ‡ <b>TVIVITL</b> § <b>VMLKKK</b> ¶	Constitutive? F-spondin ligand binding?	Forms transcription complex with FE65 and TIP60? (FOICD)
APLP1 and APLP2	Unknown physiological role (cell adhesion?)	ND	Constitutive?	Forms transcription complex with FE65 and TIP60? (FOICD)
N-cadherin	Cell adhesion	ND	Membrane depolarization; NMDA-receptor activation	Promotes CBP degradation† (FOICD)
E-cadherin	Cell adhesion	<b>LQIPAILGTILGGILALLILILLLLFL</b> RRR	STS-induced apoptosis; ionomycin treatment	Disassembly of adherens junctions* (FOC)
CD44	Cell adhesion	<b>IPFWLIILASLLA</b> ‡ <b>LALILAVCI</b> § <b>AVNSR</b> ¶	TPA and ionomycin treatment; cell scraping	Enhances TRE-mediated transcription* (FOICD)
CSF1 receptor	Protein tyrosine kinase	ND	PMA, LPS	ND
DCC	Netrin-1 receptor (axon guidance)	ND	Constitutive?	ICD-Gal4 fusion construct activates Gal4-reporter-gene expression* (FOICD)
Delta	Notch ligand	ND	Notch binding?	Downregulates Notch signalling?† (FOC)
ERBB4	Growth-factor-dependent receptor tyrosine kinase	ND	Ligand binding	Mediates apoptosis in serum-deprived cultured cells in response to ligand* (FOICD)
LRP	Endocytic receptor	ND	PMA treatment	LRP ICD antagonizes APP-ICD-Gal4 fusion-construct activity* (FOICD)
Jagged	Notch ligand	ND	Constitutive?	Downregulates Notch signalling? (FOC); modulation of AP1-mediated transcription* (FOICD)
Nectin-1 $\alpha$	Adherens-junction formation	ND	TPA treatment	Remodelling of cell junctions?† (FOC)
Notch-1-4	Signalling receptor	<b>PSQLHLMYVAA</b> ‡ <b>AAPVLLPFVGGV</b> § <b>VLLSRKR</b> ¶	Ligand binding	Transcriptional regulation*** (FOICD)
p75	Neurotrophin co-receptor	<b>TDNLIPVYCSILAAV</b> ‡ <b>VGLVAYIAFKRW</b> §	TPA and PMA treatment	Regulation of heteromeric receptor formation?† (FOC); transcriptional regulation?† (FOICD)
Syndecan-3	Cell-surface proteoglycan co-receptor	<b>RKEVLVAVIYGGVYGALFAAFL</b> ‡ <b>VILLIYRM</b> §	Ligand binding	Regulation of CASK localization* (FOC)

**Table 1.3  $\gamma$ -secretase substrates and their proposed biological functions**

\* Transmembrane domain sequences are shown in bold. ‡ Major middle transmembrane cleavage sites. § A $\beta$ 42-producing site (increasingly used in Alzheimer's disease). || Cleavage sites that release intracellular domains. ¶ Determined by overexpression assays. # Determined using  $\gamma$ -secretase inhibitors. \*\* Determined using *in vivo* genetic models. AP1, activator protein-1; APLP, amyloid-precursor-like protein; APP, amyloid precursor protein; CASK, calmodulin-dependent serine kinase; CBP, CREB (cAMP responsive- element-binding protein)-binding protein; CSF1, colony-stimulating factor-1; DCC, deleted in colorectal cancer; ICD, intracellular domain; LPS, lipopolysaccharides; LRP, low-density-lipoprotein receptor protein; ND, not determined; NMDA, N-methyl-D-acetate; PMA, phorbol-12-myristate-13-acetate; STS, staurosporine; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element. Adapted from (131).



The  $\gamma$ -secretase enzyme complex has several substrates other than APP including the notch receptor 1 (NOTCH1), the notch ligands delta-like protein (DSELT1) and Jagged2 (JAG2), v-erg-a erythroblastic leukaemia viral oncogene homologue 4 (ERBB4) and others listed in Table 1.3 (129;131). PS1 and PS2 have also been shown to interact with MOCA (modifier of cellular adhesion),  $\beta$ -catenin and proteins involved in cell death, calcium metabolism and cell adhesion (132). Mutations in PS1 have been closely associated with familial AD.



**Figure 1.6 Schematic diagram of Presenilin 1**

(a) Presenilin 1 (PS1), which contains 467 amino acids, has eight transmembrane domains, with a large cytoplasmic loop between the sixth and seventh transmembrane domains. PS2 has a similar structure, but contains 448 amino acids. (b) The large cytoplasmic loop of PS1 comprises residues 265–381 (residues given in single-letter code). The endoproteolytic cleavage site (which generates the two stable fragments) and a putative caspase cleavage site are shown. (c) The binding site for  $\beta$ -catenin has been mapped to residues 331–351, within the large cytoplasmic loop, whereas the MOCA-binding site spans the junctional region between the loop and transmembrane domain 7 (residues 375–396). MOCA, 'modifier of cellular adhesion'. After (132).

The PS1 protein is represented in Figure 1.6 (132). The 8 trans-membrane spanning regions are shown with key residues including the endoproteolytic, caspase,

$\beta$ -catenin binding and MOCA binding sites. While PS1 is not the sole protein of the  $\gamma$ -secretase complex, mutations in PS1 are closely associated with AD incidence (133).

PS1 knockout mice have an embryonic lethal phenotype (134) similar to that seen in NOTCH1 knockout mice (135) indicating that  $\gamma$ -secretase cleavage of NOTCH1 is essential in development. PS2 and PS1 are 67% identical (136), are membrane bound proteins with eight predicted transmembrane (TM) regions and a hydrophilic loop of approximately 120 amino acids between the 6<sup>th</sup> and 7<sup>th</sup> TM domains (Figure 1.6). The majority of early onset Familial Alzheimer's Disease (FAD) linked mutations occur as autosomal dominant mutations within PS1. Two in-frame deletions, two splicing mutations and over 70 missense mutations have been found distributed throughout the PS1 protein compared to only 6 missense mutations for PS2. Whilst there is a clear understanding of the genetics of the presenilins their biological function in normal neuronal cells remains unclear though the interaction of PS1 and PS2 with several proteins indicates a diversified function for these proteins and for the  $\gamma$ -secretase complex.

### 1.3.5 $\alpha$ -secretase

The identity of the  $\alpha$ -secretase has not yet been confirmed but three members of the ADAM (A Disintegrin And Metalloproteinase Domain) family of proteases, ADAM-10, ADAM-17 (TACE or Tumour Necrosis Factor-Alpha Converting Enzyme) and ADAM-9, have been proposed as  $\alpha$ -secretases (137). In particular ADAM10 has been shown to have  $\alpha$ -secretase activity *in vivo* where the expression of a catalytically inactive ADAM-10 mutant led to increased plaque size and number in double transgenic mice (138). In addition APP, BACE1 and ADAM10 have been shown to co-localise in human cortical neurones (139).

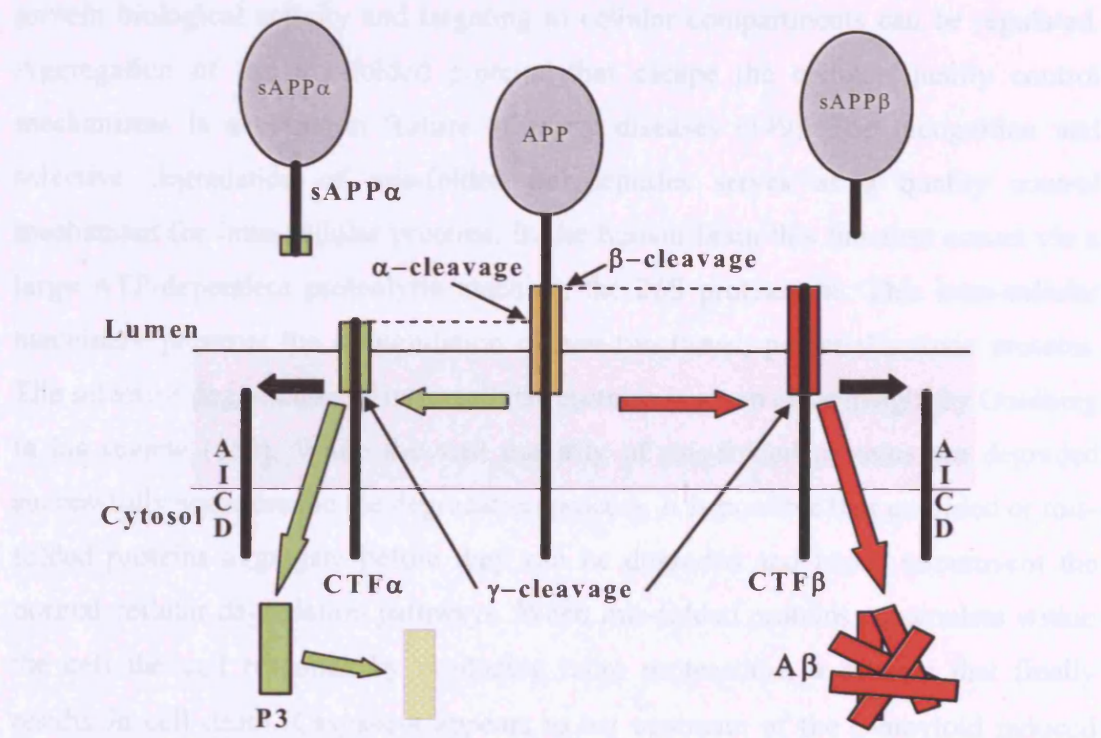
ADAM9 mRNA is ubiquitously expressed in human tissues (140) while ADAM10 mRNA expression is restricted in humans to kidney, spleen, lymph node, thymus, liver, bone marrow, and brain (141;142). ADAM17 mRNA expression is found to be highest in macrophages (143). The ADAM9 mRNA expression in human brain is higher than ADAM10 and ADAM17 mRNAs suggesting that ADAM9 mainly plays a role as "constitutive" and/or "regulated"  $\alpha$ -secretase (144). ADAM9 also has a splicing variant, which lacks the carboxyl-terminus, and the secreted short



form of ADAM9 also has an  $\alpha$ -secretase-like activity toward APP (145). Both ADAM10 (146) and ADAM 17 (147) knockout mice exhibit embryonic and perinatal lethality respectively. This lethality precludes the examination of the function of these proteins in older mice when amyloid plaques are forming however, when a dominant negative ADAM10 mutant transgenic mouse was crossed with an APP transgenic mouse, the resulting offspring had a reduced amyloid burden compared to the parental APP mouse line indicating the ADAM10 at least is anti-amyloidogenic *in vivo* (138). The evidence seems to point to a functional overlap between these 3 zinc metalloproteinases with some redundancy in  $\alpha$ -secretase activity depending on cell type.

### 1.3.6 APP Processing Pathway

The APP processing pathway explains the way in which the APP protein is acted upon by the secretase enzymes and either enters an amyloidogenic or non-amyloidogenic processing pathway (Figure 1.7). These processing events are crucial to the development of AD.



**Figure 1.7 APP Processing pathway**

APP = Amyloid Precursor Protein, sAPP $\alpha$  = soluble APP $\alpha$ , sAPP $\beta$  = soluble APP $\beta$ , CTF $\alpha$  = c-terminal  $\alpha$ , CTF $\beta$  = c-terminal  $\beta$ , P3 = P3 fragment, A $\beta$  =  $\beta$ -amyloid fragment, AICD = APP Intracellular Domain, Green = non-amyloidogenic pathway, red = amyloidogenic pathway.

Initially APP is cleaved by  $\beta$ - or  $\alpha$ -secretase in the extra-cellular region. Subsequently the residual C-terminal stub is cleaved by  $\gamma$ -secretase. An additional, novel  $\delta$ -secretase cleavage has been proposed located 12 residues from the N-terminus of the  $\beta$ -amyloid domain (148).  $\alpha$ -secretase cleavage is the initial event in the non-amyloidogenic processing of APP leading to the formation of the P3 fragment which is degraded within the cell. However cleavage by  $\beta$ -secretase initiates the amyloidogenic processing of APP leading to the formation of a 4 kD protein called  $\beta$ -amyloid. These form amyloid plaques which are one of the classic lesions definitive of AD.

### **1.3.7 Protein Mis-folding**

Subsequent to translation most proteins undergo tertiary processing before they adopt their quaternary structure and are able to perform the function for which they were transcribed. Protein folding and unfolding are crucial ways in which protein biological activity and targeting to cellular compartments can be regulated. Aggregation of the mis-folded proteins that escape the cellular quality control mechanisms is a common feature of many diseases (149). The recognition and selective degradation of mis-folded polypeptides serves as a quality control mechanism for intra-cellular proteins. In the human brain this function occurs via a large ATP-dependent proteolytic machine, the 26S proteasome. This intra-cellular machinery prevents the accumulation of non-functional, potentially toxic proteins. The selective degradation of intra-cellular proteins is given clear insight by Goldberg in his review (150). While the vast majority of mis-folded proteins are degraded successfully some escape the degradation process. It is possible that unfolded or mis-folded proteins aggregate before they can be degraded and hence circumvent the normal cellular degradation pathways. When mis-folded proteins accumulate within the cell the cell responds by producing more proteasome, a process that finally results in cell death. Caspase-4 appears to act upstream of the  $\beta$ -amyloid induced endoplasmic reticulum stress (ER stress) response to unfolded proteins. Thus Caspase-4 activation may mediate neuronal cell death in AD (151). Selkoe (82) gives an insight to systemic, organ restricted and intra-cellular amyloidoses. He states that



time, rather than old age, is the primary determinant for many amyloidoses. This is exemplified in a striking manner by Down Syndrome suffers who, carrying an additional copy of the amyloid precursor protein due to a trisomy of chromosome 21, may form A $\beta$  aggregates in the brain as early as aged 10. In familial AD, patients with mis-sense mutations in APP or  $\gamma$ -secretase may also accumulate  $\beta$ -amyloid in a reduced timescale. However the time needed for intra-cellular mis-folded proteins to overcome the proteasome is clearly reflected by the prevalence of AD in the aged population.

Recent research has pointed very strongly to the cytotoxicity in AD being due to the metastable intermediates of the fibrillogenic process rather than the stable deposits found in the plaques themselves. The concentration of oligomer species of A $\beta$  rather than amyloid plaque number gives a better correlate with decreases in synaptic terminal number in APP mouse models (152). Reassuringly, this correlation is also seen in humans where the memory and cognitive deficits of patients with Mild Cognitive Impairment is strongly linked to increased soluble cerebral A $\beta$  concentrations (153;154).

### **1.3.8 Plaques**

Amyloid plaques are the extra-cellular deposits of the fibrillar form of  $\beta$ -amyloid protein. Circulating  $\beta$ -amyloid in the blood can diffuse across the blood-brain barrier and aggregate into fibrillar forms which become deposited as amyloid plaques in the brain. Plaque deposition is initiated by extracellular accumulation of the longer A $\beta$ 42 peptide. This form of  $\beta$ -amyloid readily aggregates and is deposited as discrete amyloid plaques as it precipitates out of solution. The shorter A $\beta$ 40 peptide also accumulates in amyloid plaques after the initial seeding by A $\beta$ 42. While the plaques themselves are extra-cellular and non-toxic, the  $\beta$ -amyloid peptide is neuro-toxic (155) and hence amyloid plaques are surrounded by neuritic cells and dead cell bodies. The formation and maturation of plaques and neurofibrillary tangles contribute to the neuronal cell loss and brain shrinkage seen with the terminal decline of demented AD patients.

### 1.3.9 Tangles

Neurofibrillary tangles (NFTs) are formed from the pathogenic deposition of polymeric forms of the micro-tubule associated protein tau (MAPT or simply  $\tau$ ). The protein  $\tau$  is essential in the shaping and reshaping of cells. The normal function of  $\tau$  is to stabilise microtubule assembly and allows for new units of tubulin to be assembled on cell spindle fibres (156). Studies have shown that the  $\tau$  found in AD brains is highly phosphorylated and is found in the paired helical filaments (PHFs) (157) that are a major component of NFTs (158). Mutations in  $\tau$  can lead to hyperphosphorylation of the protein by kinases which dramatically alters its function and results in the formation of PHFs (159). Mutations in the  $\tau$  gene can also alter the stability of the mature protein by the inclusion or exclusion of exon 10 and the microtubule binding domains that it encodes thus affecting microtubule assembly and filament formation (72). In addition a recent report by Alonso *et al* (160) suggests that mutations in  $\tau$  may cause the molecule to be more susceptible to hyperphosphorylation leading to neurodegeneration by becoming 'toxic' to the microtubule system as it sequesters normal  $\tau$ .

While amyloid plaques are found during the post mortem of brains from non-demented patients, NFTs are a stronger correlate of memory impairment and disease progression in AD than are plaques and the development of NFT lesions allows the delineation of 6 stages of disease progression (161). The first two stages are present in clinically silent cases and NFTs are limited to trans-entorhinal regions. In stages 3 and 4 NFT lesions are seen in limbic regions and are presented as incipient AD. Finally fully developed AD defines stages 5 and 6 and NFT lesions extend to neocortical regions.

Mutations in  $\tau$  have been identified with fronto-temporal dementia (162) and NFTs are also present in a spectrum of diseases collectively called tauopathies (163) in which tangles are associated with neurodegeneration in the absence of plaques (164). These findings suggest that tangles are the main neurodegenerative factor in AD and not plaques. Whether AD is a type of tauopathy that includes plaques or is an amyloidopathy that includes tangles is the subject of much discussion. An understanding of the interaction between plaques and tangles in AD may be revealed by studying the activity of kinases on APP and  $\tau$ . An interesting hypothesis that may provide a unifying mechanism for tangle and plaque deposition has been proposed to

include the Notch and Wnt signalling pathways (165). Essentially PS1, a component of the  $\gamma$ -secretase complex that contributes to  $\beta$ -amyloid formation, cleaves a protein involved in cell fate determination, Notch, to generate a Notch intracellular domain (NICD) fragment. NICD may regulate GSK3 $\beta$  activity (a kinase that phosphorylates  $\tau$ ) via the dishevelled protein possibly involving protein kinase C. Dishevelled is known to be the target of Wnt-1, the *Drosophila* homologue of which, wingless, is involved in cell fate decisions and pattern formation in flies. Thus PS1 activity can contribute to  $\beta$ -amyloid production and plaque formation via APP processing and to  $\tau$  phosphorylation and tangle formation via a number of cellular kinases.

Contained within the genomic  $\tau$  locus is a discrete gene named Saitathin (STH) (166). STH is located in the intron between exons 9 and 10 of the human  $\tau$  gene. It is intronless and encodes a 128 amino acid protein with no clear homologues. STH tissue expression is similar to that of  $\tau$  and a Q7R polymorphism in the protein is over-represented in the homozygous state in late onset AD subjects. No clear function has been assigned for STH though it does contain putative phosphorylation sites that suggest a possible role in  $\tau$  phosphorylation and AD.

#### ***1.4 Clinical Interventions in Alzheimer's Disease***

Not only does Alzheimer's Disease afflict those who develop the disease, it also has a major impact on the people who care for affected individuals (167-169). It is estimated that 50% of those over 80 years of age and 10% of those over 65 years of age will develop the disease (170;171). These figures translate the estimated incidence of new cases to be 4.6 million for AD and 25 million for dementia worldwide in 2000. With the increase in the elderly worldwide population the estimate of dementia prevalence increases to 63 million in 2030 and 114 million in 2050 (172). The current incidence of AD places an enormous emotional burden on caregivers as well as a financial burden on state and private health care arrangements. The predicted increase in AD prevalence will only exacerbate these burdens and makes the search for an effective therapy more imperative.

In order to diagnose accurately the disease several methods of assessing people suspected of having a neurodegenerative disorder have been developed. These can be divided into assessments of behaviour including the cognitive state of the

person, their functional abilities and their general overall condition as well as biochemical and imaging assessments of clinical markers of disease state. In addition an index to assess the condition of the affected individual's caregivers has been developed. An examination of the methods of assessment has been valuable in order to better convey the impact that this disease has on AD patients and those that love them. The following descriptions of assessment methods are not comprehensive but they do serve to illustrate the effects of the disease.

#### **1.4.1 Prediction and Assessment of Alzheimer's Disease**

A definitive diagnosis of AD can only be given post mortem using current technologies. The presence of amyloid plaques, neurofibrillary tangles and neurodegeneration in the brains of patients post mortem are the hallmarks of the disease. AD is often diagnosed in primary care when concerned relatives approach practitioners for help with elderly relatives who appear to have changed personality and have begun to lose their memory. Generally the initial assessments of AD in subjects that present with memory impairment are behavioural. While individual tests of cognition have their own merit no one test has been shown to be better than the other. One of the tests devised is the Alzheimer's Disease Assessment Scale, Cognitive Subsection method (ADAS-Cog) (173), which tests memory, orientation, praxis and language to assess cognitive function. A scoring scale of 0 to 70 is used and most normal patients score 5 to 10 while severely demented patients score 70. Another method is the Blessed Information-Memory Concentration Test (174) which examines short term memory, concentration, spatial ability (orientation) and verbal responses. The Cambridge Neuropsychological Test Automated Battery (CANTAB) test (175) is a computer automated battery of neuropsychological tests, for use in accurate, sensitive cognitive assessment. The Clinical Dementia Rating Scale (CDR) (176) is used to gauge AD progression in clinical and longitudinal dementia studies. A widespread method is the Mini-Mental State Examination (MMSE) (177;178). The MMSE is a brief, quantitative measure which is used to test the cognitive state of adults. It can be used to screen for cognitive impairment, estimate the level of impairment at a specific time and to follow the course of mental decline over time.

In addition to cognitive testing, the function of AD patients is used to assess disease state. These include the Functional Assessment Questionnaire (FAQ) (179),

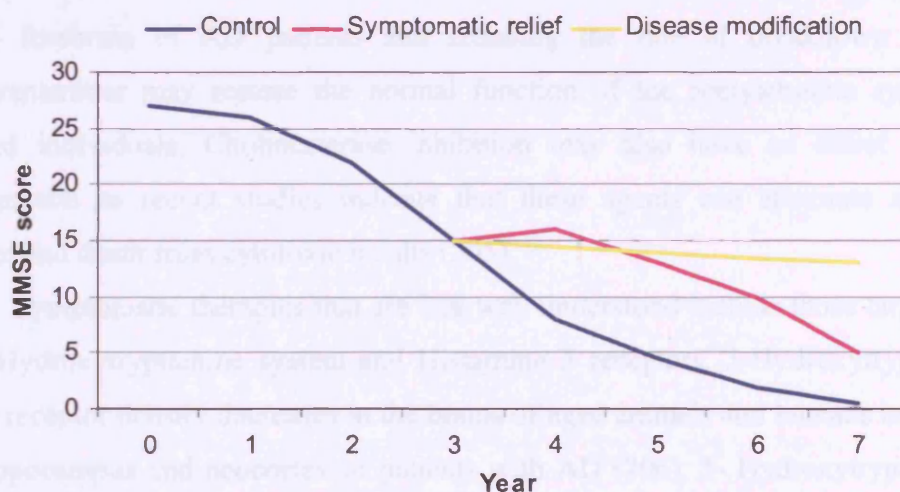
which is designed for community studies of normal aging and mild senile dementia, the Instrumental Activities Of Daily Living (IADL) and the Physical Self Maintenance Scale (PSMS) (180), both of which assess the ability of a patient to carry out normal daily activities such as going outside, paying bills, feeding, bathing, etc. and finally the Progressive Deterioration Scale (PDS) (181) looks at the quality of life experience and is assessed by the caregiver. Besides cognitive and functional assessments various global assessments of the patient have been devised. These are the Clinical Global Impression Of Change (CGIC) (182); the Clinician Interview-Based Impression (CIBI) (183) and the Global Deterioration Scale (CDS) (184). Finally there are 2 caregiver based assessments. These are the Behavioural Pathology In Alzheimer's Disease Rating Scale (BEHAVE-AD) (185) and the Neuropsychiatric Inventory (NPI) (186). The assessments provide an indication of the impact that the patient has on those that look after the individual. An excellent overview of these and many more rating scales used in dementia research is given by Lon Schneider (Alzheimer Insights Online 2(3) Oct 1996). The plethora of test scales used to assess AD patients and caregivers is a clear indication of the devastating nature of this disease and the widespread collateral burden of care that it places on loved ones (187).

A number of tests are used to diagnose the disease including volumetric analysis of the brain ventricles with MRI (188) which can also be predictive of AD (189), imaging amyloid pathological lesions in the brain with Positron Emission Tomography (PET) scanning (190;191), biomarkers of AD (192) and harvesting cerebrospinal fluid to test for the presence of brain specific proteins diagnostic of neurodegenerative disease (193;194).

Peripheral indicators such as elevated cortisol levels have also been linked to cognitive decline (195). Studies have shown that the function of the hypothalamus and Hypothalamic-Pituitary-Axis (HPA) is impaired in AD patients and HPA over activity may be responsible for the non cognitive behavioural disturbances seen in demented patients (196). Indeed the detrimental effects of chronic glucocorticoid hypersecretion occurring through over-activation of the HPA have been seen in obesity, AIDS dementia and depression as well as in AD (197).

In the century following Alois Alzheimer's publication extensive research has been carried out on all aspects of the disease. An effective cure or comprehensive symptomatic relief still remain elusive despite the many millions of pounds spent on

research and development into a therapy for AD. Individuals that increase mental stimulation during youth and midlife tend to be protected from AD in later years (198). While inactivity may be a risk factor for AD or may be reflective of a very early sub clinical feature of the disease, these data suggest that daily recreational activities such as playing bingo or completing crosswords may be effective in reducing the chances of developing AD. An interesting corroboration to this report is the finding that when mice with elevated  $\beta$ -amyloid protein and plaques were exposed to an enriched environment including coloured tunnels, toys and chewing material over a 5 month period the  $\beta$ -amyloid load and plaque number was reduced (199). Calorie restriction has been shown to attenuate  $\beta$ -amyloid deposition in APP transgenic mice (200) hinting at a simple protective treatment against AD by reduction of calorie intake, however starvation in mice has been shown to increase  $\tau$  hyperphosphorylation at sites predominantly hyperphosphorylated in AD (201) hence promoting the development of NFTs. Finally animals that were allowed access to a running wheel in their cage for 1 month showed a decrease in  $\beta$ -amyloid load pointing to voluntary exercise as a prophylactic in AD (202). While these reports indicate potential lifestyle changes that may help to treat or prevent AD, current therapies are directed at alleviating the symptoms of the disease (symptomatic treatments) or altering the underlying disease pathology (disease modifying treatment).



**Figure 1.8 Predicted change in MMSE score in response to treatment**  
After Gauthier *et al* (203).

The goal of symptomatic therapies is to alleviate the symptoms of the disease and slow cognitive decline. While this strategy may be easier to achieve and indeed is the only proven method of treating AD, it does not alter the disease progression. Symptomatic treatment is represented by the red line in Figure 1.8. Disease modifying therapies seek to interfere with the underlying disease pathology, halting disease progression and hence stop the cognitive decline. This strategy is represented by the yellow line in Figure 1.8. Disease modification can be further split into therapeutic agents that seek to prevent the formation of the pathogenic proteins, namely  $\beta$ -amyloid and hyperphosphorylated  $\tau$ ; seek to prevent the deposition of the pathogenic lesions that develop in AD, namely plaques and tangles; and those that treat the many other components of disease pathology that arise from the presence of these lesions in the brain.

#### **1.4.2 Symptomatic Treatments for Alzheimer's Disease**

The ways in which current symptomatic treatments work have been extensively studied. Aricept™ is marketed by Lilly and is the current market leader for the treatment of Alzheimer's Disease (204). Aricept™ is one of a class of treatments called acetyl cholinesterase inhibitors. These compounds act by inhibiting the enzyme acetyl cholinesterase which catalyses the breakdown of the neurotransmitter acetylcholine. Clear dysfunction is seen in the acetylcholine system in the forebrain of AD patients and reducing the rate of breakdown of this neurotransmitter may restore the normal function of the acetylcholine system in affected individuals. Cholinesterase inhibition may also have an effect on AD pathogenesis as recent studies indicate that these agents can attenuate neuronal damage and death from cytotoxic insults (205).

Symptomatic therapies that are less well understood include those targeted at the 5-Hydroxytryptamine system and Histamine 3 receptors. 5-Hydroxytryptamine (5HT) receptor density decreases in the brains of aged animals and humans including the hippocampus and neocortex of patients with AD (206). 5- Hydroxytryptophane treatment of aged rats increases cognitive performance in a test of spatial learning and memory (207) pointing to the 5HT system as an avenue therapeutic intervention in AD. Receptor agonists have been developed for 5-HT<sub>1A</sub> (208;209), 5-HT<sub>4</sub> (210)

and 5-HT<sub>6</sub> (211;212). In addition selective histamine H<sub>3</sub> receptor antagonists have been shown to be effective in treating cognitive disorders (213).

### 1.4.3 Disease Modification Treatments for Alzheimer's Disease

Disease modifying therapies for AD have been the subject of intense research by academic and industrial laboratories. However a limited understanding of the disease biology has hampered progress towards an effective treatment. Even when a disease modifying therapy is identified, the very long time course of the disease makes a total treatment strategy difficult to plan. Nevertheless a number of potential leads have been followed in the search for a way of modifying AD progression (214).

Preventing the formation of the neurotoxic  $\beta$ -amyloid peptide is an active topic for researchers as it provides a treatment that interferes with the disease progression. The major targets for preventing the formation of  $\beta$ -amyloid are the agonism of  $\alpha$ -secretase and the inhibition of  $\gamma$ - or  $\beta$ -secretases. Increasing the activity of  $\alpha$ -secretase with an agonist is problematic as normal physiological processing ensures that the bulk of APP enters the non-amyloidogenic  $\alpha$ -secretase cleavage pathway so that only small changes may be brought about. This pathway can be stimulated via M1 muscarinic receptor agonists which may function as cognitive enhancers and may influence disease progression if they impact on  $\beta$ -amyloid production (215). While a number of enzymes have been identified at candidates for  $\alpha$ -secretase (see 1.3.5), the full effect of increasing the activity of these proteins, unrelated to APP cleavage, needs to be investigated as part of a screening cascade for compound development.

Inhibition of  $\gamma$ - and  $\beta$ -secretase is an attractive strategy as precedent exists for this target class with the marketing of successful inhibitors of HIV protease. As stated earlier, PS1 is a component of the  $\gamma$ -secretase complex.  $\gamma$ -secretase has been shown to cleave NOTCH1 and both NOTCH1 and PS1 knockout mice are embryonic lethal (see 1.3.4). Hence when efforts at developing  $\gamma$ -secretase inhibitors led to dose dependant toxic effects, probably Notch related (216),  $\gamma$ -secretase inhibition did not look to be a viable target. An alternative approach to  $\gamma$ -secretase inhibition was indicated when very high doses of non-steroidal anti-inflammatory drugs (NSAIDs) were found to be able to modulate  $\gamma$ -secretase activity such that the



longer, more aggregation prone species of  $\beta$ -amyloid, A $\beta$ 42, was reduced. This result was seen in conjunction with an increase in the less aggregating A $\beta$ 38 species of  $\beta$ -amyloid and no effect on NOTCH1 cleavage (217). These results paved the way for  $\gamma$ -secretase inhibition as a therapy with more potent  $\gamma$ -secretase modulators that are selective against Notch1 cleavage. Recent reports indicated that a potent  $\gamma$ -secretase inhibitor, LY-411575, was able to lower CSF and brain  $\beta$ -amyloid, but elevated plasma  $\beta$ -amyloid at low doses (218). The use of NSAIDs as a potential  $\gamma$ -secretase inhibition therapy for AD must be viewed with some caution however as a number of compounds have been found to increase A $\beta$ 42 production (219).

The finding that BACE1 is an aspartyl protease allowed the prospect of  $\beta$ -secretase inhibition as a viable disease modification strategy for AD (see 1.3.2). BACE1 knockout mice are viable and fertile (see paragraph 1.5) though they have subtle changes in anxiety and cognition (see chapters 2 and 3 of this thesis). These findings indicate that BACE1 is a viable target for inhibition and several BACE1 inhibitors have been described (220;221). BACE2 is a homologue of BACE1 and deleting BACE2 in addition to BACE1 in mice does not appear to alter the phenotype beyond that seen in BACE1 mice alone (see chapter 4 of this thesis). Consequently BACE2 inhibition is not actively pursued as a drugable target for AD (222). While inhibition of BACE1 appears to be very attractive as a target, the active site of the enzyme is unusually large and thus represents a challenge to medicinal chemists to develop small molecules that cross the blood brain barrier and inhibit enzyme activity, though progress has been made (223). A novel strategy for BACE1 inhibition was recently suggested with the effective development of antibodies that inhibit the interaction between BACE1 and APP (224). This  $\beta$ -amyloid immunotherapy approach is attractive and several groups have demonstrated the efficacy of this approach as a  $\beta$ -amyloid lowering therapy (225). In addition data showing altered anxiety and cognition suggest a role for BACE1 independent of that of a  $\beta$ -secretase. Further work is required to define any other potential roles for BACE1 in humans and appropriate screening cascades designed to screen out any potential deleterious effects of BACE1 inhibition as a therapeutic strategy.

The  $\beta$ -amyloid fragment of APP has a propensity to aggregate into oligomers and can undergo further aggregation to create the fibrils that are found in amyloid plaques. One suggested method of a therapeutic intervention in AD is to interfere

with the mechanism by which the pathogenic proteins fold and become aggregated. Potential events that are initiated by mis-folded and aggregated proteins include excitotoxicity, increased cellular oxidative stress, impaired cellular antioxidant functioning and/or impaired molecular chaperone and protein folding mechanisms (226). Anti-fibrillation therapies have met with limited success. The search for a fibrillation inhibitor has focussed on inhibition of  $\beta$ -amyloid fibrillation *in vitro* using compounds identified from screening compound banks. Data suggest that these inhibitor compounds do not all bind to a common single site on  $\beta$ -amyloid. (227). In addition the mechanism by which these protein-protein interactions occur is not clearly understood. In the absence of a clear mechanism of action the design of small molecule inhibitors of fibrillation using structural activity relationship modelling is extremely difficult. In order to develop an effective anti-fibrillation therapy, a small molecule would need to interfere with the specific protein-protein interaction of  $\beta$ -amyloid peptide binding to  $\beta$ -amyloid peptide, a strategy that has a track record of failure in the industry (228). A vital component of  $\beta$ -amyloid aggregation is zinc. The antibiotic Clioquinol has been reported to lower  $\beta$ -amyloid burden in APP transgenic mice by copper and zinc chelation (229). This strategy for anti-fibrillation of  $\beta$ -amyloid was progressed to the clinic with subtle positive effects in a subset of patients warranting further studies (230).

An alternative therapeutic approach to the treatment of AD is to try and clear the  $\beta$ -amyloid peptide from the brain and blood of patients. Vaccination of APP overexpressing mice with aggregated A $\beta$ 42 resulted in a surprising improvement in plaque load, neuritic dystrophy and gliosis in the mice with no apparent ill effects (231). Inoculation with fragments of  $\beta$ -amyloid elicits an antibody response in the body and levels of circulating amyloid are reduced. This effect is thought to occur via one of two mechanisms. Either  $\beta$ -amyloid vaccination stimulates microglia activation and plaques are cleared via Fc mediated phagocytosis (232;233), or  $\beta$ -amyloid antibodies induce a change in the  $\beta$ -amyloid equilibrium between the CNS and plasma acting as a peripheral sink for  $\beta$ -amyloid (234;235). Human clinical trials of amyloid vaccination have resulted in the reduction of CNS amyloid plaques. While this technique has proved efficacious as a disease modifying therapy for AD side effects, including neuroinflammation, have led to a cessation of the clinical trial and a return to the basic research into the mechanisms thought to underlie the

process (236;237). New hopes for this approach have been raised with the finding that AD patients with serum antibodies against  $\beta$ -amyloid plaques show improved cognition and slowed disease progression (238;239).

Therapeutic interventions aimed at preventing the formation of the pathogenic forms of  $\tau$  include stopping  $\tau$  hyperphosphorylation, promoting  $\tau$  dephosphorylation, preventing PHF formation and aggregation, promoting PHF disaggregation, clearing PHF from the CNS and ameliorating the consequences of PHF formation in neuronal cells. As  $\tau$  is phosphorylated by multiple phosphatases and kinases (240;241) an effective  $\tau$  specific therapy that targets  $\tau$  phosphorylation may prove to be problematic. However recent reports indicate that stabilising microtubules may be an effective treatment.  $\tau$  transgenic mice that were dosed with a microtubule binding drug showed an improvement in the impaired fast axonal transport and motor function seen in untreated mice (242).

Additional strategies have been suggested as potential avenues by which AD may be treated. Miller *et al* (243) have employed strand interference RNA (siRNA) technology to develop short hairpin RNA (shRNA) plasmids that can interfere with the production of  $\tau$  and APP *in vitro*. This technology has been shown to silence the expression of genes *in vivo* (244) and has obvious application for the inhibition of pathogenic protein production in AD patients. In particular the size of the BACE1 enzyme active site has proved difficult to inhibit with small molecule compounds and siRNA technology may yield an effective therapeutic agent (245). The main caveats to this technology are efficient delivery, sustained cell specific silencing of targeted genes and the possible side effects that such silencing may have on normal cell function (246).

Epidemiological studies have shown that people taking cholesterol biosynthesis inhibitors (statins) have a reduced risk of developing AD. While this mechanism is not fully understood, Sidera *et al* (247) showed that a cholesterol transport inhibitor, U18666A, produced a change in the glycosylation state of BACE1, possibly by interfering with the trafficking and recycling of lipid rafts. Lipid rafts are a cellular compartment where BACE1, APP, Presenilin and  $\beta$ -amyloid have been shown to co-localise. Interestingly there is epidemiological evidence that increased dietary intake of the omega 3 (n-3) polyunsaturated fatty acid (PUFA) decosahexanoic acid (DHA) is associated with a reduced risk of AD (248;249).

When fed with a DHA enriched diet, the  $\beta$ -amyloid burden in aged APP transgenic mice was decreased (250) suggesting utility for increased dietary DHA as a protective therapy for AD.

Medicinal use of the Ginkgo Biloba tree (Maidenhair tree in English) has been traced back to 1280 AD during the Chinese Yuan dynasty. An extract from the tree, EGb 761, has been shown to improve cognitive performance in aged animals possibly acting via mitochondrial stimulation (251). This demonstrates a natural product approach to finding an effective treatment for AD.

### ***1.5 Transgenic and Knockout Mouse Models***

Transgenic models are those in which a protein from the gene of interest is over-expressed. This over-expression can be controlled in a temporal or spatial manner with appropriate use of DNA regulatory sequences. Knockout models are generated by removing the endogenous protein expression from a gene of interest.

Several amyloid depositing mouse models have been generated using different promoters, gene constructs and APP mutations linked to familial forms of AD (252-254). In all of these models there is an age dependent increase in brain  $\beta$ -amyloid levels and concomitant amyloid plaque deposition associated with astrogliosis, microgliosis and the appearance of dystrophic neurites (252;253;255). There are also reports of reduced synaptophysin staining in some of these models, notably the PDAPP mouse model (152;256) and some neuronal loss in the APP23 mouse model (257), although none of these mouse lines demonstrate a convincing level of neuronal degeneration. Behaviourally, several of the APP transgenic lines exhibit deficits in learning and memory (258-262) lending support to the notion that elevated  $\beta$ -amyloid levels and plaque deposition can cause behavioural changes despite a lack of neuronal loss (263).

The molecular, enzymatic and cell biological characterisation of BACE1 has significantly increased our understanding of the role of this enzyme in  $\beta$ -amyloid generation and presented the scientific community with a new therapeutic target for the treatment of AD. Further validation of BACE1 as a key enzyme in the generation of  $\beta$ -amyloid has been made possible using mice lacking BACE1. In such animals there is a complete inhibition of  $\beta$ -amyloid production, either from murine APP or in animals which are crossed with mice over-expressing APP (264). Furthermore

removal of endogenous BACE1 by interbreeding BACE1 KO mice with a transgenic mouse line carrying mutant human APP (Tg2576) rescues the cognitive deficit seen in the APP mouse line alone demonstrating that it is the  $\beta$ -amyloid peptide rather than APP overexpression itself that leads to cognitive deficits (263). BACE1 knock-out (KO) mice are viable and fertile, with an absence of any deleterious or adverse findings (264-266). This is in sharp contrast to mice lacking presenilin-1, one of the components of the  $\gamma$ -secretase enzyme complex (130;267), which have an embryonic lethal phenotype (134). These observations, coupled with the clear correlation between  $\beta$ -amyloid burden and cognitive decline (268), provide good evidence that inhibition of BACE1 could be of benefit to AD patients and potentially be less deleterious than approaches targeting inhibition of  $\gamma$ -secretase.

In addition to the neuropathological features described above, the brains of AD patients are deficient in a multitude of neurotransmitter systems. These include acetylcholine and glutamate, upon which current symptomatic therapies such as cholinesterase inhibitors and NMDA antagonists are based, as well as noradrenalin, serotonin, and neuropeptides such as somatostatin and neuropeptide Y (269-271). The evaluation of neurotransmitter deficits in transgenic mouse models of AD has only recently been undertaken. Several reports have suggested the existence of cholinergic deficits in APP transgenic mice (272;273). In addition, elevated levels of neuropeptides such as galanin, neuropeptide Y, cholecystokinin and enkephalin have been reported in aged brains of PDAPP mice (274). However the extent to which these changes are common to all mouse models of AD and their direct relevance to changes observed in AD patients remain to be investigated.

## ***1.6 The need for a new model***

The BACE1 mouse models described in this thesis have been generated in order to examine the effect of increasing or eliminating BACE1 expression *in vivo*. While it is clear that BACE1 is the key enzyme in the development of the  $\beta$ -amyloid plaques found in Alzheimer's Disease, other functions may yet be revealed. Research investigating the numerous models of human APP overexpression has not yielded an effective treatment for AD and a comprehensive examination of BACE1 transgenic and knockout mouse models may increase the likelihood of the discovery of such a

treatment as well as point towards any potential problems associated with BACE1 therapeutic agents. By combining these BACE1 models with other transgenic and knockout mice, of both single and compound gene targets, the altered BACE1 protein expression can be put into context alongside existing models.

The mouse models described in this thesis are those in which altered BACE1 gene expression is a primary feature of the mouse model, as with endogenous BACE1 expression removed in a so called knockout mouse (BACE1 KO) or where an expression cassette has been introduced to express human BACE1 protein in a normal mouse genetic background in a so called transgenic mouse (BACE1 Tg). In addition to these mouse lines the BACE1 KO mouse line has been crossed with a BACE2 KO mouse line to generate a BACE1/2 double KO mouse line and the BACE1 Tg mouse line has been crossed to an APP Tg mouse line, named TAS10 (275), to form a double transgenic line named BACTAS. In order to make a comparison of increased expression of key enzymes involved in plaque formation, a second double transgenic line carrying human APP and PS1 transgenes, named TASTPM (276), was included the tests.

## **1.7 Objectives of Thesis**

This thesis sets out to investigate the biology of BACE1 using *in vivo* mouse models. Therapies that interfere with the activity of  $\beta$ -secretase may have unknown consequences *in vivo* as the full extent of the functions of this enzyme are not clearly defined. The focus of this thesis is on the analyses of those behavioural phenotypes which are most obvious in human AD patients. Investigating the consequence of removing or increasing the protein on behaviour in a living organism will enable a deeper understanding of the other functions of BACE1 and any potential side effects of  $\beta$ -secretase therapy may be identified and screened out during the preclinical development of the therapeutic agent.

*Chapter 2 - BACE1 transgenic  
mice are bold and knockout  
mice are timid*

## 2.1 Introduction

Genetically modified mouse lines were generated in which (i) endogenous BACE1 was removed to produce a BACE1 knockout (KO) mouse line and (ii) human BACE1 was inserted to produce BACE1 transgenic (Tg) mouse lines. In addition BACE1 Tg mice were crossed with APP Tg mice to produce the BACTAS double Tg mouse line (dTg). These KO, Tg and respective wild type (wt) control mice were subjected to a screening battery of tests designed to examine basic differences in their anatomy and behaviour. Consistent with other reports the BACE1 KO and single Tg mouse lines were viable, fertile and healthy. However some subtle differences were seen in basic measures of physiology and in exploratory behaviour in these lines.

Both the Tg and KO mouse lines described in this thesis were generated by incorporating a LacZ reporter gene such that endogenous gene expression and inserted transgene expression could be easily followed by staining for  $\beta$ -galactosidase expression. Once generated the different lines of BACE1 Tg and KO mice were bred in numbers sufficient to produce enough animals for the studies. In order to investigate the effects of increasing or removing expression of BACE1 in mice the different lines were tested in the SHIRPA protocol. SHIRPA is an acronym standing for SmithKline Beecham, Harwell, Imperial College of St. Mary's, Royal Holloway College Phenotype Assessment and was developed by Rogers *et al* (277). This test was selected as it is sensitive to both gross and subtle behavioural deficits in mice and has been used routinely to discover novel phenotypes in mice generated from random (278) and targeted (279) mutant mouse research programmes.

The SHIRPA protocol includes three levels of behavioural assessment known as primary, secondary and tertiary screens. Behavioural data from the primary and secondary SHIRPA screens are presented in this chapter and tertiary SHIRPA screening behavioural data is presented in chapter 3. The primary SHIRPA screen comprises 39 basic measures to provide a behavioural, neurological and anatomic profile of each mouse. The secondary SHIRPA screen consists of four, more complex, tests which are; the Rotarod Assessment of Sensorimotor Deficits in which balance and co-ordination are tested; the Spontaneous Locomotor Activity (LMA) test which records the movement of each mouse in a home cage; the Holeboard Test of Exploratory Behaviour which records the number of times each mouse places its



head in one of 12 holes and the Hotplate Test of Thermal Nociception in which sensitivity to thermal pain is recorded.

## **2.2 *Materials & Methods***

### **2.2.1 *Generation of mouse models***

Manipulations of the BACE1 gene to generate the mouse models are detailed in appendix 7.1. In brief BACE1 KO mice were generated by replacing Exon 1 of the endogenous BACE1 locus with a LacZ reporter gene in a DNA targeting vector. This vector was then introduced into 129Sv Embryonic Stem (ES) cells by electroporation. The cloned DNA integrated with the ES cell genome by homologous recombination and positive recombination events were selected for with positive selectable markers. ES cells carrying the correctly integrated engineered murine genomic DNA were introduced into the blastocoel of 3.5 day old C57Bl/6J host mouse embryos by micro-injection. Those embryos surviving the procedure were returned to the uterus of pseudopregnant foster mice and allowed to develop to term. The relative contribution of the genetically engineered ES cell genome to the genome of the resultant [C57Bl/6 x 129OlaSv]hybrid pups was assessed by the proportion of light (ES Cell) and dark (host embryo) coat colour. Male offspring with approximately a 50:50 split of coat colour were selected to breed with wt females to generate subsequent generations. Mice with a heterozygous deletion of BACE1 were bred to wt mice on a C57Bl/6 background sub-strain over 5 generation of breeding to generate a KO mouse line on an isogenic C57Bl/6J background sub-strain. Finally these N6 mice were interbred to generate mice used in subsequent experiments.

BACE1 Tg mice were generated by constructing an expression cassette in which human BACE1 cDNA expression is under the control of a calmodulin kinase 2 promoter (280). An Internal Ribosomal Entry Site (IRES) element was introduced down stream of the BACE1 cDNA followed by a LacZ reporter gene. The IRES element enabled the generation of a bicistronic mRNA which is translated to give 2 protein products (281). Thus the expression of human BACE1 protein *in vivo* is indicated by the blue stain produced when the BACE1 Tg mouse tissue is stained for  $\beta$ -galactosidase activity. This expression cassette was micro-injected into the male pronucleus of a single-celled C57Bl/6 mouse embryo. Embryos surviving the

procedure were placed into the oviduct coils of a pseudo-pregnant female mouse via the infundibulum and allowed to develop to term. As C57Bl/6J donor embryos were used the offspring from the recipient host female were all isogenic for the C57Bl/6J sub-strain. 5 mice carrying the transgene were selected for breeding the N1F1 generation of mice produced from this breeding were analysed for BACE1 transgene expression. Mice with high and low expression levels were selected for further breeding to generate the heterozygous BACE1 Tg mice used for experimentation.

### **2.2.2 BACE1 compound mouse lines**

The BACTAS dTg mice were generated by inter-breeding BACE1 Tg mice with an APP Tg mouse line previously established in the laboratory (275). The APP mouse used employed the Thy1 promoter to drive expression the 695 amino acid isoform of APP carrying the Swedish (APP<sub>KM670/671NL</sub>) mutation, hence the abbreviation Thy1-APP-Swedish (TAS). Both of these mouse lines were isogenic for the C57Bl/6J mouse sub-strain.

TASTPM dTg mice were obtained from a colony previously established within GSK (276). These mice were generated by interbreeding the TAS mice with a Tg line carrying a M146V mutant Presenilin 1 gene under the control of the human Thy1 promoter. The abbreviation is derived from the first transgene components, Thy1-Presenilin-Mutant (TPM).

### **2.2.3 Behavioural analysis**

The SHIRPA screen (277) was used to assess the behavioural phenotype of the heterozygous Tg mouse line expressing the human BACE1 transgene on an inbred C57Bl/6 background and a homozygous BACE1 KO line on a mixed genetic background. Each study cohort consisted of 10 male and 10 female Tg or KO animals and 10 male and 10 female wild-type littermates. Mice were housed individually and maintained under a standard 12-hr light: dark cycle with food and water available *ad libitum*. Testing began when the mice were between 6 and 7 weeks of age. Similarly the BACTAS mice were tested in the primary SHIRPA screen though the age of testing was approximately 25 months.

The primary SHIRPA screen recorded 39 basic measurements starting with unobtrusive observation of the mice in a viewing jar (Table 2.1) through observation in an arena (Table 2.2), above the arena (Table 2.3) and ending with supine restraint

(Table 2.4). In addition, Rotarod, 30-minute locomotor activity, holeboard exploration and the thermal analgesia hotplate tests were included in the secondary SHIRPA screen.

### **SHIRPA – Primary screen protocol**

A full description of the test is detailed by Rogers *et al* (277) however a brief description of the scoring scales used is provided here to facilitate an overall impression of the phenotypes observed. The equipment used and a description of the protocol is listed in Appendix 7.2.

### **Behaviour Recorded in the Viewing Jar.**

The animal was placed in the viewing jar for 5 min. This was located on top of a grid which was suspended above a piece of white paper. The following behaviours were recorded without disturbing the animal. Incidents of atypical or stereotyped behaviour and convulsions were recorded separately

**Table 2.1 Primary SHIPRA Scoring in viewing jar**

	<b>Score</b>	<b>Observed Behaviour</b>
Body position	0	Completely flat
	1	Lying on side
	2	Lying prone
	3	Sitting or standing
	4	Rearing on hind legs
	5	Repeated vertical leaping
Spontaneous activity	0	None
	1	Casual scratch, groom, slow movement
	2	Vigorous scratch, groom, moderate movement
	3	Vigorous, rapid/dart movement
Respiration	0	Gasping, irregular
	1	Slow, shallow
	2	Normal
	3	Hyperventilation
Tremor	0	None
	1	Mild
	2	Marked

### Behaviour Recorded in the Arena.

A metal plate was inserted under the viewing jar and the animal transferred and briskly dropped onto the centre of the floor of the arena without being handled. The stop watch was started and the immediate reaction to the new environment was recorded.

**Table 2.2 Primary SHIRPA Scoring in Arena**

	Score	Observed Behaviour
Transfer arousal	0	Coma
	1	Prolonged freeze, then slight movement
	2	Extended freeze, then moderate movement
	3	Brief freeze (few sec), then active movement
	4	Momentary freeze, then swift movement
	5	No freeze, immediate movement
	6	Extremely excited ("manic")
Locomotor activity	n	Number of squares crossed in 30 seconds
Palprebral closure	0	Eyes wide open
	1	1/2 closed
	2	Closed
Piloerection	0	None
	1	Coat stood on end
Startle response (90dB sound from clickbox 30 cm above arena)	0	None
	1	Preyer reflex (backwards flick of pinnae)
	2	Jump <1cm
	3	Jump >1cm
Gait	0	Normal
	1	Fluid but abnormal
	2	Limited movement only
	3	Incapacity
Pelvic elevation	0	Markedly flattened
	1	Barely touches floor
	2	Normal (3mm elevation)
	3	Elevated (>3mm elevation)
Tail elevation (during forward motion)	0	Dragging
	1	Horizontally extended
	2	Elevated / Straub tail
Touch-escape (Finger stroke from above)	0	No response
	1	Mild (escape response to firm stroke)
	2	Moderate (rapid response to light stroke)
	3	Vigorous (escape response to approach)
Positional passivity (struggle response to sequential handling)	0	Struggles when held by tail
	1	Struggles when held by neck (finger grip, not scruffed)
	2	Struggles when laid supine (on back)
	3	Struggles when held by hind leg
	4	No struggle

## Behaviour Recorded above the Arena

The mice were held above or on the arena either by scruffing or tail suspension and the following behaviours recorded.

**Table 2.3 Primary SHIRPA Scores on or above the Arena**

	Score	Observed Behaviour
Trunk Curl	0	Absent
	1	Present
Limb grasping	0	Absent
	1	Present
Other (see comments)	0	Absent
	1	Present
Visual placing (extension of forelimbs when lowered to wire grid)	0	None
	1	Upon nose contact
	2	Upon vibrissae contact
	3	Before vibrissae contact (18mm)
	4	Early vigorous extension (25mm)
Mouse removed from arena and tail gripped between thumb and forefinger. Mouse was lowered and allowed to grip the grid then a gentle backwards pull was applied		
Grip strength (horizontal pull applied To animal on wire grid)	0	None
	1	Slight grip, semi-effective
	2	Moderate grip, effective
	3	Active grip, effective
	4	Unusually effective
Body tone (compress sides of animal between thumb and index finger)	0	Flaccid, no return of cavity to normal
	1	Slight resistance
	2	Extreme resistance, board-like
The proximal part of the inner canthus was lightly touched with the tip of the fine wire probe.		
Pinna reflex (ear retraction upon light tactile stimulation)	0	None
	1	Active retraction, moderately brisk flick
	2	Hyperactive, repetitive flick
The cornea was lightly touched with the side of the fine wire probe.		
Corneal reflex (eye-blink response to light tactile stimulation with stylet)	0	None
	1	Active single eye-blink
	2	Multiple eye-blink
Hind limb lifted clear of grid and mid digit of hind foot gently compressed with fine forceps		
Toe pinch (gentle compression of mid- digit of hind foot)	0	None
	1	Slight withdrawal
	2	Moderate withdrawal, not brisk
	3	Brisk, rapid withdrawal
	4	Very brisk with repeated extension and flexion
Animal held above the wire by tail suspension and lowered to allow the forelimbs to grip the wire		
Wire manoeuvre (animal allowed to hang by forelegs from wire)	0	Active grip with hind legs
	1	Difficulty of grasp with hind legs
	2	Unable to grasp with hind legs
	3	Unable to lift hind legs, falls within 10 sec
	4	Falls immediately

The mice are scruffed firmly and the following are observed during supine restraint

**Table 2.4 Primary SHIRPA Scores recorded during Supine Restraint**

	Score	Observed Behaviour
Body weight		Grams
Body length (tip of nose to base of tail)		mm
Skin colour (colour of plantar surface and digits of forelimbs)	0 1 2	Blanched Pink Bright, deep red
Heart rate (felt by palpation below sternum)	0 1 2	Slow, bradycardia Normal Fast, tachycardia
Limb tone (resistance to gentle and finger tip pressure on plantar surface of left right hind paw)	0 1 2 3 4	No resistance Slight resistance Moderate resistance Marked resistance Extreme resistance
Abdominal tone (palpation of abdomen)	0 1 2	Flaccid, no return of cavity to normal Slight resistance Extreme resistance, board-like
Lacrimation	0 1	None Present
A dowel rod was gently inserted between the teeth at the side of the mouse's mouth		
Salivation (area of wetness in sub-maxillary area)	0 1 2	None Slight margin of sub-maxillary area Wet zone entire sub-maxillary area
Provoked biting	0 1	Absent Present
Mouse held by the tail and flicked backwards through the air such that it performs a backward somersault when released. Landing position was observed.		
Righting reflex (somersaulted onto table by tail)	0 1 2 3	No impairment Lands on side Lands on back Fails to right when placed on back
Mouse placed into a plastic tube and turned upside down		
Contact righting reflex	0 1	Absent Present
Mouse placed on a grid and raised from horizontal to vertical then observed for 30 seconds.		
Negative geotaxis (placed on grid which is raised to vertical with animal facing floor)	0 1 2 3 4	Turns and climbs the grid Turns but then freezes Moves but fails to turn Does not move within 30 sec Falls off
Fear	1	Freezes during transfer arousal
Irritability	1	Struggles during supine restraint
Aggression	1	Provoked biting or attack
Vocalisation	1	Provoked during handling

**Table 2.5 Secondary SHIRPA screen**

Behaviour	Summary Protocol
<b>Holeboard Test of Exploratory Behaviour</b>	Mouse placed in Holeboard Exploration arena and allowed to explore for 10 minutes. Exploratory behaviour recorded as the number of infrared beam breaks by the mouse (each time it placed its head in a hole). There are 12 holes in the floor in a regularly spaced but asymmetric pattern.
<b>Rotarod Assessment of Sensorimotor Deficits</b>	Mouse was placed on the surface of a rotating drum which accelerates from 4 to 40 rpm over 5 minutes. Each mouse receives 3 trials.
<b>Spontaneous Locomotor Activity (LMA) Test</b>	Mouse was placed in a home cage, lined with sawdust only, which was placed in the recording apparatus. LMA recorded as the number of infrared beam breaks in 30 minutes.
<b>Hotplate Test of Thermal Nociception</b>	Mouse was placed on a hot plate analgesia meter set at 50.0°C and was enclosed within an annulus of Perspex. Thermal nociception measured as the latency to the first sign of nociception, i.e. fanning or licking of paws.

### 2.2.4 Determination of LacZ expression by $\beta$ -galactosidase assay

Perfusion fixed adult brains from both the human BACE1 Tg overexpressing mice and from mice heterozygous for the targeted mutation were dissected, equilibrated in 30% sucrose, embedded in OCT cryoprotectant (Agar Scientific) and frozen. Cryostat sections (20-25 $\mu$ m) were prepared, mounted onto slides and incubated for between 1 hour and overnight at 37°C in X-Gal solution containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% NP-40, 0.01% sodium deoxycholate and 2 mM MgCl<sub>2</sub> in PBS. After staining, sections were washed twice in PBS for 5 min, counterstained in 1% Orange G, rinsed in distilled water, dehydrated through a methanol series (50%, 70%, 100%; 5 mins each) and then washed in histoclear twice prior to mounting in DPX mounting medium.

### 2.2.5 Western Blot Analyses

Synaptosomes were prepared from mouse brains using the method of Gray and Whittaker (282). Samples from 2 KO, 3 Tg and 2 wt control animals were analysed by SDS-PAGE. To visualise full length APP 10% Tris-Glycine polyacrylamide gels (Novex) were used. Protein was transferred to a nylon membrane (Hybond) and probed with an anti-BACE1 mouse monoclonal antibody (9B21) and HRP-conjugated detection antibody (Amersham). The carboxyterminal fragments were separated on a 12% NuPage BisTris gel (Invitrogen) and probed with rabbit polyclonal Ab54 which recognises the extreme carboxyterminus of APP. Actin was detected using a mouse monoclonal antibody (ICN). Immunoreactive bands were detected using ECL (Amersham) and Biomax film (Kodak).



### **2.2.6 Statistical Analysis**

Throughout this thesis statistical analyses were conducted using Statistica™ (Statsoft, Inc). ANOVA was performed to compare the effects of genotype on each of the behavioural variables. The differences between individual means were assessed with post hoc simultaneous comparisons using the Sheffe test. Where appropriate, non-parametric data were analysed according to the variation in the data set. Data with no variation were not analysed with a statistical test, data with variation in a binomial response were analysed with Fisher's Exact test, data with variation of more than 2 responses in only 1 group were first grouped to give a binomial set of responses and then tested using Fisher's Exact test and finally data that had multiple responses in both groups were tested with the Mann-Whitney U procedure.

## **2.3 Results**

### **2.3.1 Production of Tg and KO mice**

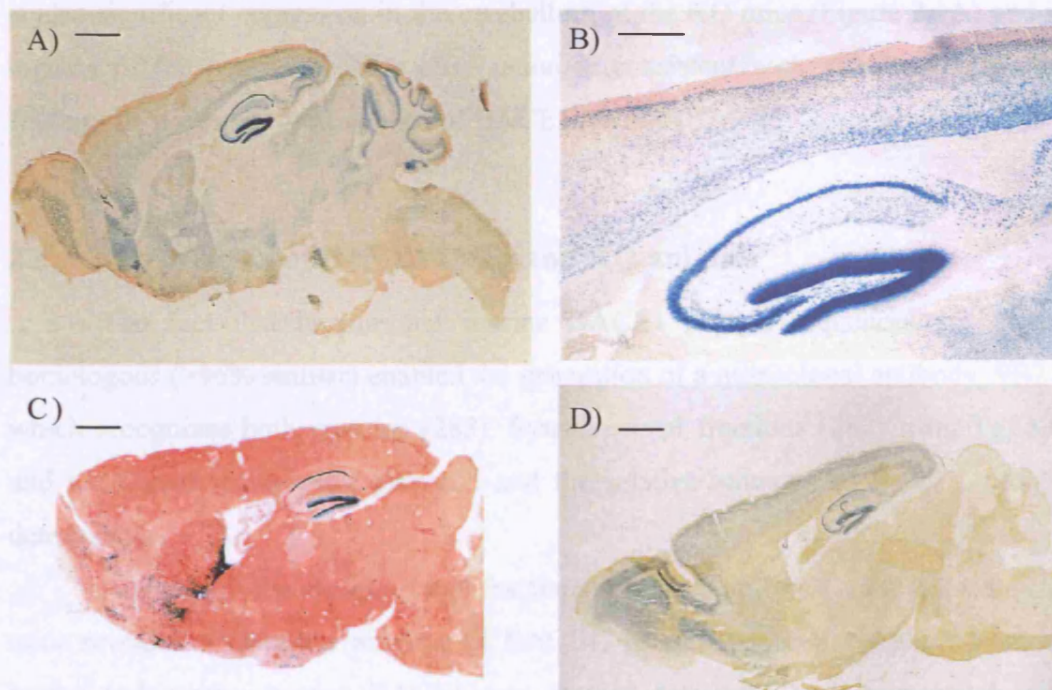
Tg mice were successfully generated from the micro-injection of the expression cassette. Two Tg lines had LacZ expression patterns that represented high (Line 4) and low (Line 17) levels of BACE1 expression in the mouse brain. Subsequent separate breeding programmes crossing these two lines to wt C57Bl/6 mice generated sufficient mice for experimentation. The BACTAS mice were successfully generated by interbreeding the BACE1 Tg mouse line with a line overexpressing human mutant APP<sub>SWE</sub> protein. The BACTAS mice were heterozygous for both transgenes and were bred to a background strain essentially isogenic for the C57Bl/6J sub strain.

Following successful integration of 129Ola/Sv embryonic stem cells injected into host C57Bl/6 blastocysts, BACE1 KO mice of the founder generation mice (those born from injected blastocysts) were produced. Breeding 5 of these male founder mice to C57Bl/6J wt mice produced offspring, denoted N1 (from the first number generation crossed to an isogenic, C57Bl/6, mouse strain). These were interbred to produce F1N1 (first Filial and first Number backcrossed generation)

mice with 25% of the resulting offspring carrying homozygous disruption of the endogenous BACE1 locus on a [C57Bl/6x129Ola/Sv] hybrid background.

### 2.3.2 Expression patterns of LacZ in Tg and KO mice

As both the Tg and KO animals were generated using a reporter construct design in which the LacZ gene was incorporated into the mouse genome, staining for the expression of LacZ enabled the expression of the human calmodulin kinase 2 promoter driven transgene or the murine BACE1 promoter driven endogenous gene to be followed. In the Tg mice  $\beta$ -galactosidase expression is localised to the hippocampus, cortex and olfactory bulb with diffuse expression in the caudate striatum (Figure 2.1).



**Figure 2.1  $\beta$ -galactosidase expression in BACE1 mouse models**

Parasagittal sections of BACE1 Tg and KO mice. BACE1 KO mouse showing  $\beta$ -galactosidase expression concentrated in the hippocampus and distributed in cortical lamina A). High magnification image of the hippocampus and cortex of a BACE1 KO animal B). BACE1 Tg mouse brain showing  $\beta$ -galactosidase expression localised to the hippocampus and caudate striatum in a low expressing mouse line C) and extending to the cortex and caudate putamen in a higher expressing mouse line D). Bars in A), C) and D) = 1.5 mm, B) = 0.5 mm.

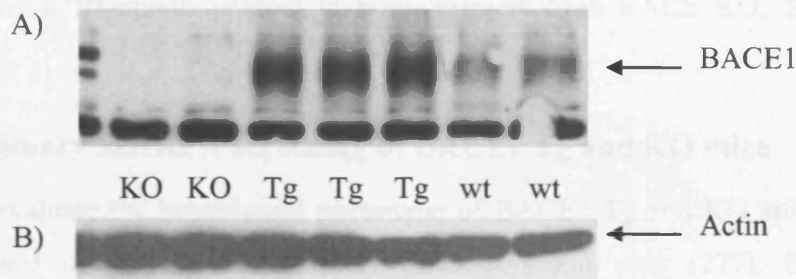
The integration of the targeting construct into the endogenous murine BACE1 chromosomal locus places the LacZ gene under the control of the BACE1 promoter, which makes it possible to visualise tissue expression from the endogenous murine BACE1 promoter by monitoring  $\beta$ -galactosidase enzyme activity or immunoreactivity. There is significant expression of  $\beta$ -galactosidase through the brain of BACE1 KO mice though it is concentrated in the hippocampus and cortex indicating the endogenous expression pattern of this gene (Figure 2.1A). Increased magnification allows visualisation of a clear laminated expression pattern in the cortex (Figure 2.1B). In mice heterozygous for the targeted mutation,  $\beta$ -galactosidase expression is localised to the hippocampus and caudate striatum in the low expressing line 17 (Figure 2.1C) and extends to the cortex, olfactory bulb, medulla and fore-brain regions in the high expressing line 4 (Figure 2.1D). Interestingly, there is also significant expression in the cerebellum of the KO mice (Figure 2.1A) and in regions of the periphery. This observation is consistent with previously reported findings of widespread expression of BACE1 mRNA (89;90).

### **2.3.3 Expression of BACE1 in Tg and KO animals**

The fact that human and murine BACE1 protein sequences are highly homologous (>96% similar) enabled the generation of a monoclonal antibody, 9B21, which recognizes both proteins (283). Synaptosomal fractions (282) from Tg, KO and wt animal brains were prepared and the relative amounts of BACE1 protein determined.

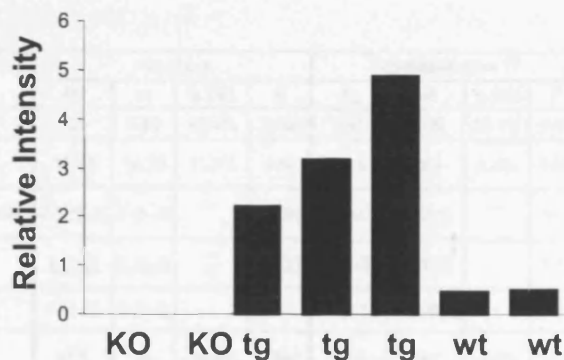
Analysis of the synaptosomal fractions from two individual KO homozygous mice revealed a complete absence of BACE1. In synaptosomal fractions from wt brains endogenous murine BACE1 was readily detectable and co-migrates with recombinant BACE1 protein isolated from HEK293 cells expressing the human BACE1 cDNA (283). The equivalent fraction from 3 separate BACE1 Tg animals indicated clear BACE1 over-expression of the human transgene (Figure 2.2A). Quantification of the western blot signal using the  $\beta$ -actin signal as loading control (Figure 2.2B) indicated that individual animals show BACE1 protein levels 4-10 fold greater than the levels of expression of murine BACE1 in wt animals (Figure 2.3). Endogenous BACE1 protein in wt mice and human BACE1 expressed in Tg mice appeared to be glycosylated to a similar degree, with both proteins migrating as

broad bands with an apparent MW of 64 kDa. Analysis of the APP cleavage products in these same fractions indicates a clear absence of carboxy-terminal fragments generated by BACE1 cleavage (CTF $\beta$ ) in the KO mice and the appearance of CTF $\delta$  (148) and elevated levels of the same fragment in Tg mice (Figure 2.4). These findings are consistent with previous reports of similar KO and Tg lines (257;265;266).



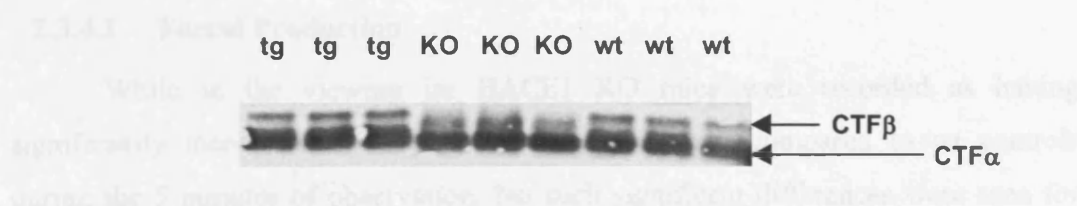
**Figure 2.2 Expression of BACE1 protein in Tg and KO mice**  
A) BACE1 (9B21) immunoreactivity. B) Actin Immunoreactivity.

Immunoblot analysis blot showing immunoreactivity to BACE1 in brain extracts from BACE KO, Tg and wild type (wt) mice using monoclonal antibodies specific to BACE1 (9B21 in figure 2.2A) or to the housekeeping protein actin (figure 2.2B).



**Figure 2.3 Densitometric analysis of BACE1 expression**

Densitometric analysis of BACE1 immunoreactivity in Tg and KO mice normalised against the  $\beta$ -actin housekeeping control for each animal



**Figure 2.4 Immunoblot examining the c-terminal APP fragments**

Immunoblot analysis with Ab54 to the c-terminus of APP showing the carboxyterminal fragments present in brain extracts from BACE KO, Tg and wt mice.

### 2.3.4 Primary SHIRPA screening of BACE1 Tg and KO mice

To evaluate the behavioural phenotype of BACE1 Tg and KO animals they were assessed in the SHIRPA battery of behavioural tests (277). Significant differences in the measures recorded during the primary SHIRPA behavioural characterisation of BACE1 KO and Tg mice are listed below (for detailed results of all the tests see appendices 7.3 and 7.4). The KO and two Tg mouse lines failed to show any significant differences in motor function as measured by either a Rotarod test of motor co-ordination or a 30 minute test of locomotor activity. As such, it can be concluded that altered motor function was not responsible for the changes observed between in the mouse lines.

Measure	Units	Knockout				Transgenic Line 17				Transgenic Line 4			
		KO	wt	% Diff.	P	Tg	wt	% Diff.	P	Tg	wt	% Diff.	P
Faeces	number	5.9	3.95	49.4%	0.02	3.45	3.05	13.1%	0.42	3.2	3.65	-12.3%	0.42
Arena Activity	squares crossed	16.05	14.35	11.8%	0.56	22.5	22.4	0.4%	0.95	17.9	22.9	-21.8%	<0.01
Wire Manoeuvre	righting ability	0.5 (0-1)	2 (1-2)	-	<0.01	0 (0-0.5)	0 (0-1)	-	0.3	0 (0-1)	0 (0-0)	-	0.29
Limb Tone	resistance	2 (1-2)	2 (2-2)	-	0.051	2 (2-2)	2 (2-2)	-	0.82	2 (2-2)	2 (2-2)	-	0.79
Provoked Biting	intensity	0 (0-0)	0 (0-0)	-	1	0 (0-1)	0 (0-0.5)	-	0.28	1 (0-1)	0 (0-0.5)	-	0.058
Holeboard Exploration	number of visits	37.1	58	-36.0%	0.048	42.6	46.7	-8.8%	0.59	78.3	59.8	30.9%	0.052

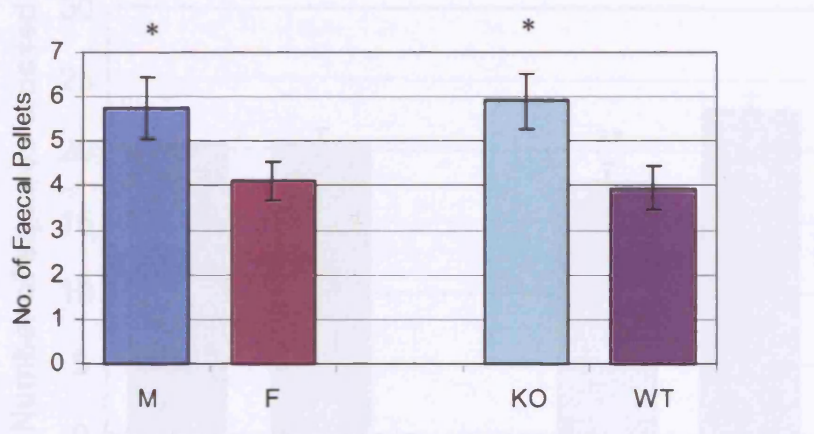
**Table 2.6 Significant differences found during primary SHIRPA**

Data are represented as mean with percent change from wt control for parametric measures or median with interquartile range for non-parametric measures. P-value calculated using 1-way ANOVA or Mann-Whitney U test as appropriate. Results are collapsed for gender. N = 20.



### 2.3.4.1 Faecal Production

While in the viewing jar BACE1 KO mice were recorded as having significantly increased faecal pellets production when compared to wt controls during the 5 minutes of observation. No such significant differences were seen for the BACE1 Tg mouse lines.



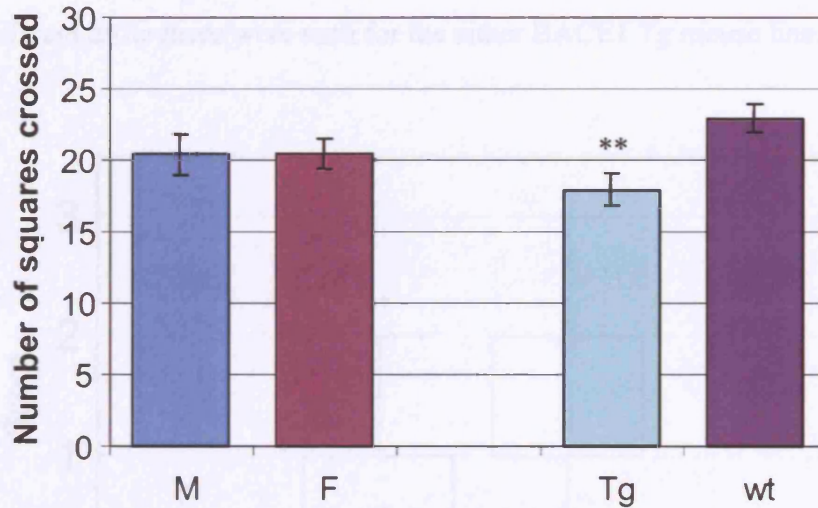
**Figure 2.5 Faecal production in BACE1 KO mice**

Bars represent mean  $\pm$  SEM. \*  $p < 0.05$

Analysis of data from the BACE1 KO mice revealed a significant effect of gender  $F(1,36) = 4.54$ ,  $p = 0.04$  and genotype  $F(1,36) = 6.34$ ,  $p = 0.02$  on faecal production with the KO mice producing 49% more faeces than controls mice during a 5-minute observation period (Figure 2.5). No significant interaction between gender and genotype was observed  $F(1,36) = 0.94$ ,  $p = 0.34$ . No significant effect of gender or genotype was seen on this measure for the high (line 4) or low (line 17) expression BACE1 Tg mice.

#### 2.3.4.2 Arena Activity

While in the arena BACE1 Tg line 4 mice crossed fewer squares than wt littermates controls during the 30 seconds of observation. No such significant differences were seen for the BACE1 Tg line 17 or BACE1 KO mouse lines.



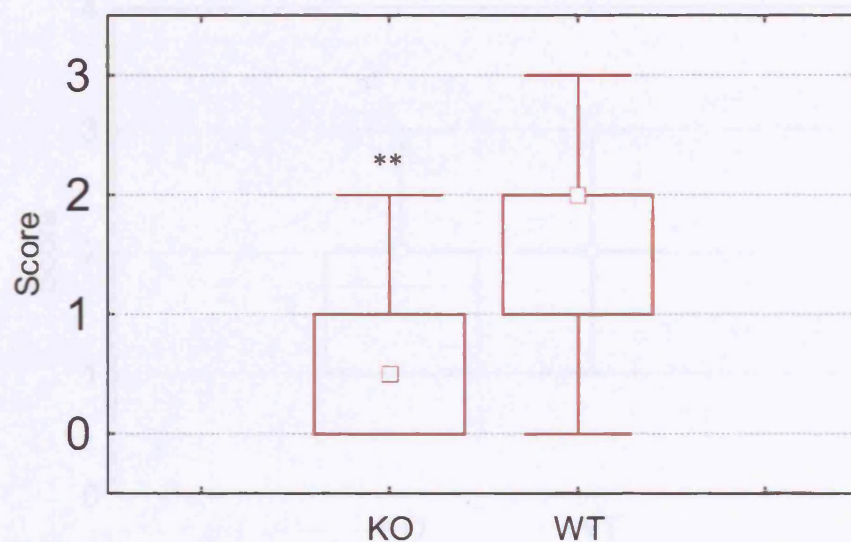
**Figure 2.6 Arena Activity of BACE1 Line 4 Tg mice**  
Bars represent mean  $\pm$  SEM. \*  $p < 0.05$

BACE1 Tg line 4 mice were significantly different from wt controls  $F(1,36) = 10.9$ ;  $p = 0.002$  with Tg mice crossing 22% fewer squares in the 30 second recording period (Table 2.6). No significant effect of gender or interaction between gender and genotype was observed in Tg line 4 and no significant effect of gender or genotype was seen for the BACE1 KO or low (line 17) expression BACE1 Tg mouse lines.



### 2.3.4.3 Wire Manoeuvre

The wire manoeuvre test examines hind limb strength and coordination. While observed above arena BACE1 KO mice performed well in this test suggesting that they had improved muscle strength and/or co-ordination over wt controls. No such significant differences were seen for the either BACE1 Tg mouse line.



**Figure 2.7 BACE1 KO mice wire manoeuvre**

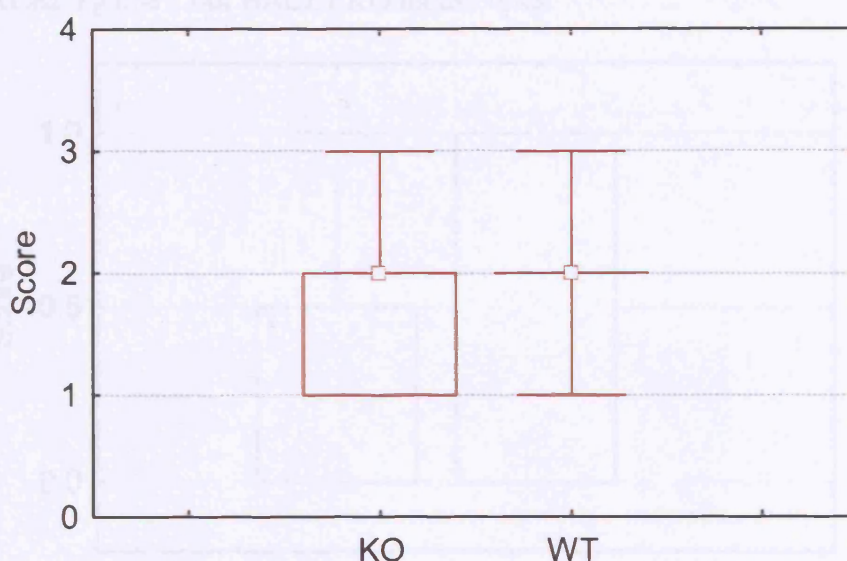
Key:  Median  25%-75%  Min-Max. \*  $p < 0.05$

BACE1 KO mice were significantly different from wt controls  $p = 0.007$  with KO mice having a median score of 0.5 compared to a wt littermate control mice a median score of 2. This result indicated the BACE1 KO mice had an improved righting ability in a wire manoeuvre test (Figure 2.7). No significant effect of gender was observed in KO mice and no significant effect of gender or genotype was seen for the BACE1 Tg line 17 or line 4 mouse lines.



#### 2.3.4.4 Limb Tone

While held in supine restraint above the arena the mice were assessed for differences in limb tone. This measure gives an overall indication of the muscle strength in a limb. Several of the BACE1 KO had a reduced limb tone compared to wt controls suggesting weaker limb muscles in these mice. No such differences were seen for the BACE1 Tg line 17 or line 4 mouse lines.



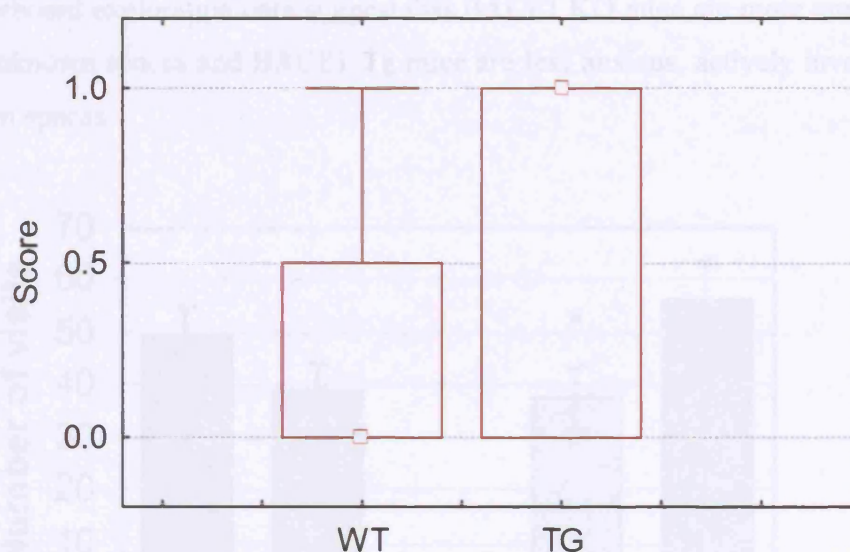
**Figure 2.8 BACE1 KO mice limb tone**

Key: □ Median    □ 25%-75%    I Min-Max

BACE1 KO mice were almost significantly different from wt controls  $p = 0.051$  with KO mice and wt littermate controls having a median score of 2 but KO mice having a lower interquartile range (1-2) than controls (2-2). A score of 2 indicates moderate resistance to gentle finger tip pressure on a hind paw for both lines of mice but the range of responses of the BACE1 KO mice included mice with slight as well as moderate resistance (Figure 2.8). No significant effect of gender was observed in KO mice and no significant effect of gender or genotype was seen for the BACE1 Tg line 17 or line 4 mouse lines.

#### 2.3.4.5 Provoked Biting

While held in supine restraint above the arena the mice were provoked into biting the end of a plastic rod by placing the rod close to their mouth. Their response to this provocation was recorded and BACE1 Tg line 4 mice were found to bite the rod more readily than wt controls indicating that these mice were more ready to respond with aggression when provoked. No such significant differences were seen for the BACE1 Tg line 17 or BACE1 KO mouse lines.



**Figure 2.9 BACE1 Tg line 4 mice provoked biting**

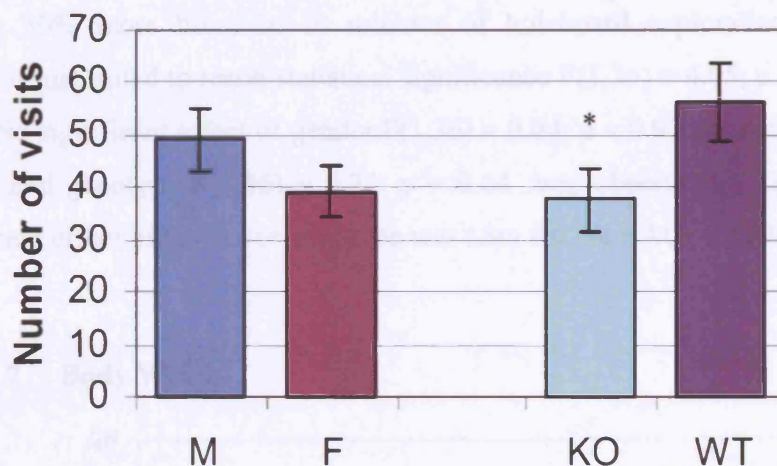
Key: □ Median    □ 25%-75%    I Min-Max

BACE1 Tg line 4 mice were almost significantly different from wt control mice  $p = 0.058$  with Tg mice having a median score of 1 compared to a control score of 0. These data indicate that the Tg Line 4 had biting response present when provoked whereas the wt controls did not (Figure 2.9). No significant effect of gender was observed in Tg line 4 and no significant effect of gender or genotype was seen for the BACE1 KO or low (line 17) expression BACE1 Tg mouse lines.



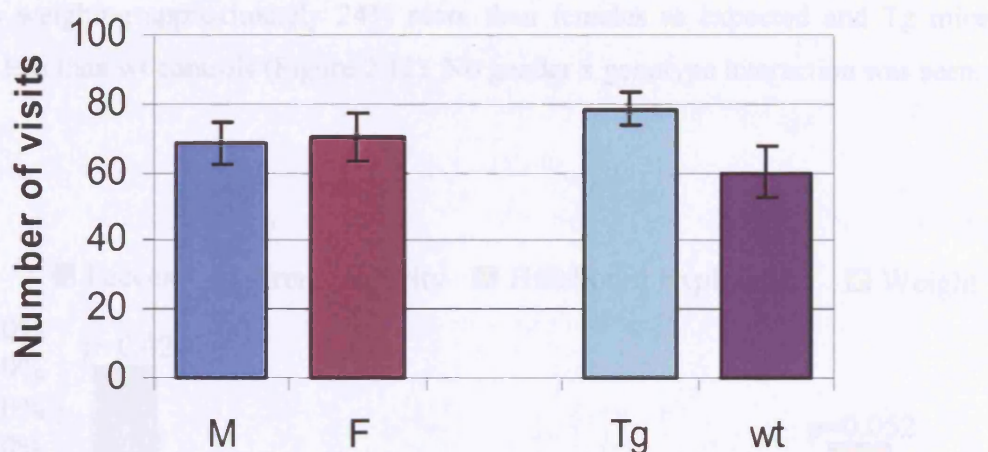
#### 2.3.4.6 Holeboard Exploration

When allowed to explore an arena with holes in the floor placed at irregular intervals for 10 minutes, BACE1 KO mice explored significantly fewer holes than wt controls and BACE1 Tg Line 4 mice explored more holes than wt controls though this measure just failed to reach significance. No such significant differences were seen for the BACE1 Tg line 17 mouse line. Differences in exploratory behaviour reflect the inquisitive nature of the mice and can be linked to differences in anxiety. The holeboard exploration data suggest that BACE1 KO mice are more anxious and avoid unknown spaces and BACE1 Tg mice are less anxious, actively investigating unknown spaces.



**Figure 2.10 BACE1 KO mouse number of visits during holeboard exploration**  
Mean  $\pm$  SEM \*  $p < 0.05$

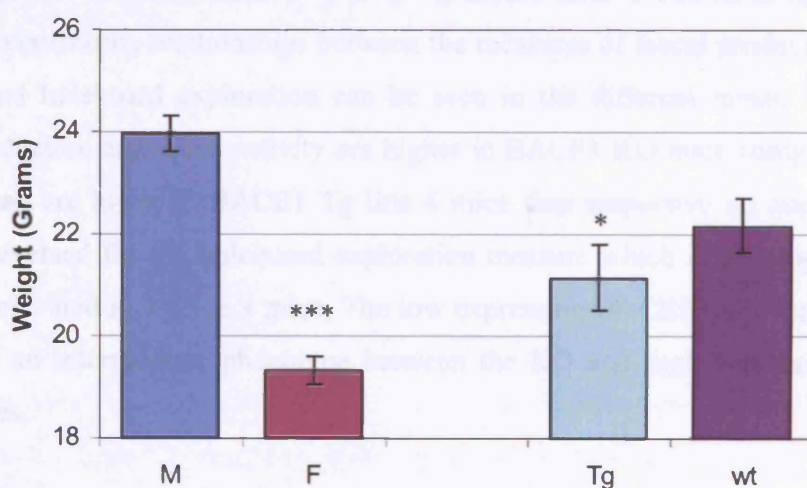
BACE1 KO mice were significantly different from wt controls  $F(1,32) = 4.24$ ,  $p = 0.048$  with the KO mice examining 33% fewer holes during 10 minutes of holeboard exploration (Figure 2.10). No significant interaction between gender and genotype was observed  $F(1,32) = 1.37$ ,  $p = 0.25$  and no significant effect of gender was seen  $F(1,32) = 1.76$ ,  $p = 0.19$ .



**Figure 2.11 BACE1 Tg line 4 mice holeboard exploration number of hole visits**  
Mean +/- SEM

BACE1 Tg line 4 mice were different from wt controls with Tg line 4 mice visiting 30% more holes in 10 minutes of holeboard exploration although this measures just failed to reach statistical significance  $F(1,36) = 4.05$ ;  $p = 0.052$  (Figure 2.11). No significant effect of gender  $F(1,36) = 0.04$ ;  $p = 0.87$  or interaction between gender and genotype  $F(1,36) = 0.23$ ;  $p = 0.64$  was observed in Tg line 4 and no significant effect of gender or genotype was seen for the BACE1 Tg line 17.

#### 2.3.4.7 Body Weight

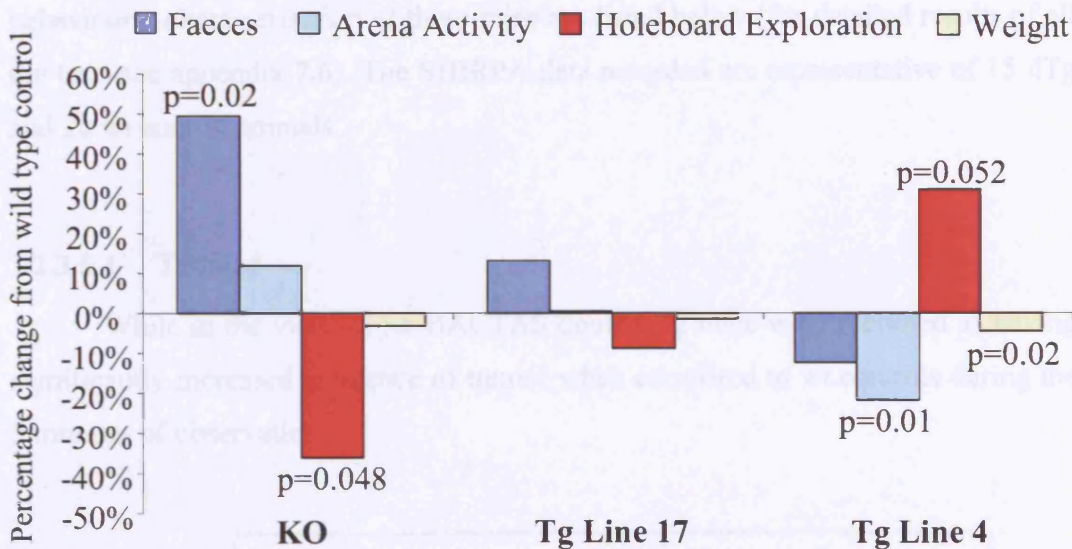


**Figure 2.12 Weight of BACE1 Tg Line 4 mice**  
Mean +/- SEM \*  $p < 0.05$ , \*\*\*  $p < 0.001$

Analysis of the weight of BACE1 Line 4 Tg mice revealed a significant effect of gender  $F(1,36) = 115.89$ ;  $p < 0.001$  and genotype  $F(1,36) = 5.58$ ;  $p = 0.024$  with



males weighing approximately 24% more than females as expected and Tg mice 4.5% less than wt controls (Figure 2.12). No gender x genotype interaction was seen.



**Figure 2.13 Summary of differences in primary SHIRPA measures**  
N = 20. Mean values. Comparison is to respective wild type controls.

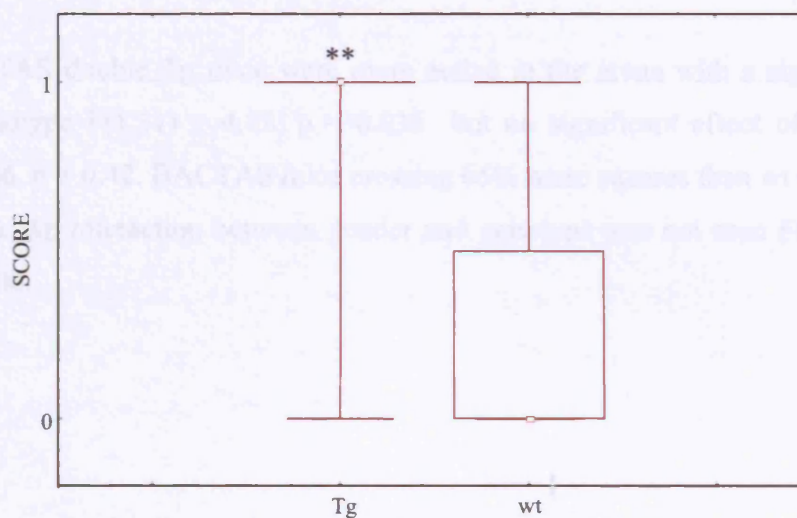
A histogram showing percentage differences relative to controls for the number of faecal pellets produced, arena activity, hole-board exploration and weight of KO, Tg Line 4 (high), Line 17 (Low) Tg mouse lines is shown in figure 2.13. Clear and contrasting relationships between the measures of faecal production, arena activity and holeboard exploration can be seen in the different mouse lines. The faecal production and arena activity are higher in BACE1 KO mice compared to wt controls and are lower in BACE1 Tg line 4 mice than respective wt controls. The result is reversed for the holeboard exploration measure which is decreased in KO mice and elevated in Tg line 4 mice. The low expressing BACE1 line 17 mouse line represents an intermediate phenotype between the KO and high expressing line 4 mouse lines.

### 2.3.5 Primary SHIRPA screening of BACTAS double Tg mice

In order to evaluate the behavioural phenotype of the BACTAS double Tg animals they were similarly assessed in the SHIRPA battery of behavioural tests. Significant differences in the measures recorded during the primary SHIRPA behavioural characterisation of these mice are listed below (for detailed results of all the tests see appendix 7.6). The SHIRPA data recorded are representative of 15 dTg and 20 wt control animals.

#### 2.3.5.1 Tremor

While in the viewing jar BACTAS double Tg mice were recorded as having significantly increased incidence of tremor when compared to wt controls during the 5 minutes of observation.



**Figure 2.14 Incidence of Tremor in BACTAS mice**

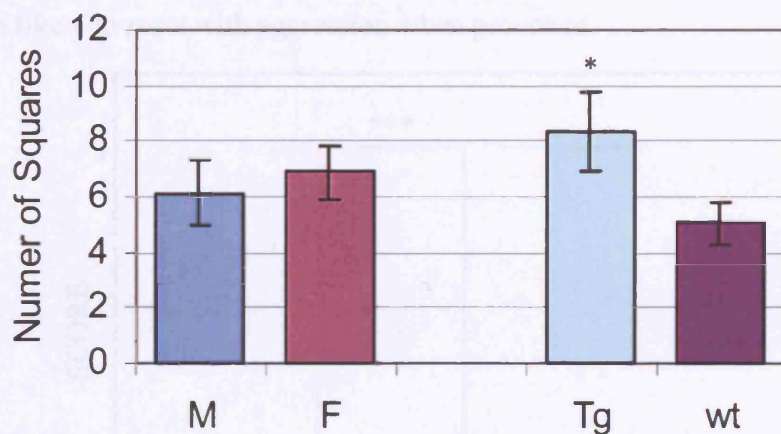
Key: □ Median    □ 25%-75%    I Min-Max. \*  $p < 0.05$

Analysis of Tremor in BACTAS mice revealed no significant effect of gender,  $p = 0.12$ , but did reveal an effect of genotype,  $p = 0.001$  (Figure 2.14) with BACTAS double Tg mice having a median score of 1 with an interquartile range of (1-1) and wt littermate control mice a median score of 0 with an interquartile range of (0-0.5) indicating the Tg mice had a mild tremor but controls did not.



### 2.3.5.2 Arena Activity

While in the arena BACTAS dTg mice were more active than wt littermate controls during the 30 seconds of observation.

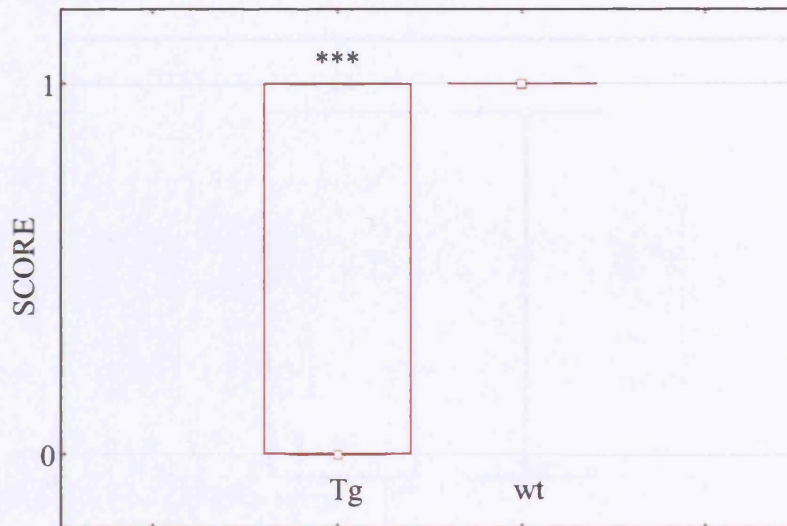


**Figure 2.15 Arena Activity of BACTAS mice**  
Mean +/- SEM. \*  $p < 0.05$

BACTAS double Tg mice were more active in the arena with a significant effect of genotype  $F(1,31) = 4.82$ ,  $p = 0.036$  but no significant effect of gender  $F(1,31) = 0.66$ ,  $p = 0.42$ . BACTAS mice crossing 65% more squares than wt controls (Figure 2.15). An interaction between gender and genotype was not seen  $F(1,31) < 0.001$ ,  $p = 1.00$ .

### Provoked Biting

While held in supine restraint above the arena the mice were provoked to bite a plastic rod held close to the mouth. The BACTAS dTg mice were found to be less likely to bite when provoked when compared to wt controls indicating that the mice were less likely to react with aggression when provoked.



**Figure 2.16 Provoked Biting of BACTAS mice**

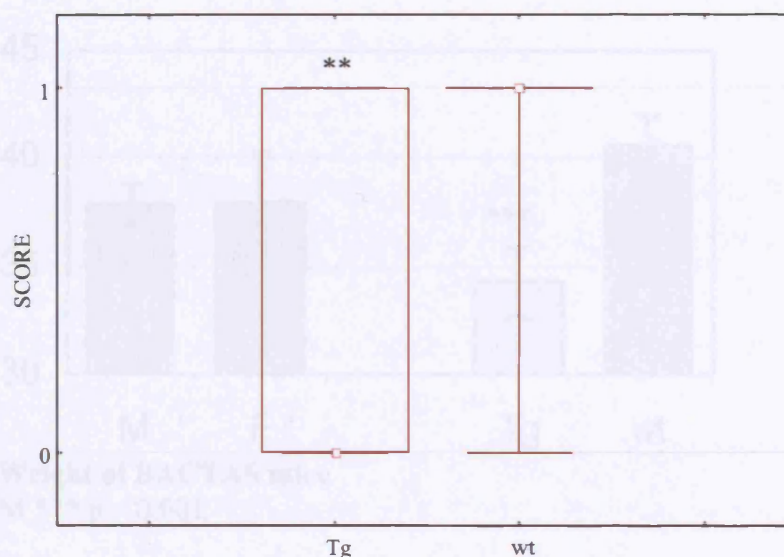
Key: □ Median ■ 25%-75% ▬ Min-Max. \*\*\*  $p < 0.001$

When provoked to bite a plastic rod a significant effect of genotype,  $p < 0.001$ , but no significant effect of gender,  $p = 0.64$  was seen (Figure 2.16). BACTAS mice had a median score of 0 with an interquartile range of (0-1) and wt littermate control mice a median score of 1 with an interquartile range of (1-1).



### 2.3.5.3 Aggression

While held in supine restraint above the arena BACTAS double Tg mice had a reduced incidence of aggressive responses than wt controls. These data are in agreement with the provoked biting data showing an overall reduced aggressive phenotype in these mice.



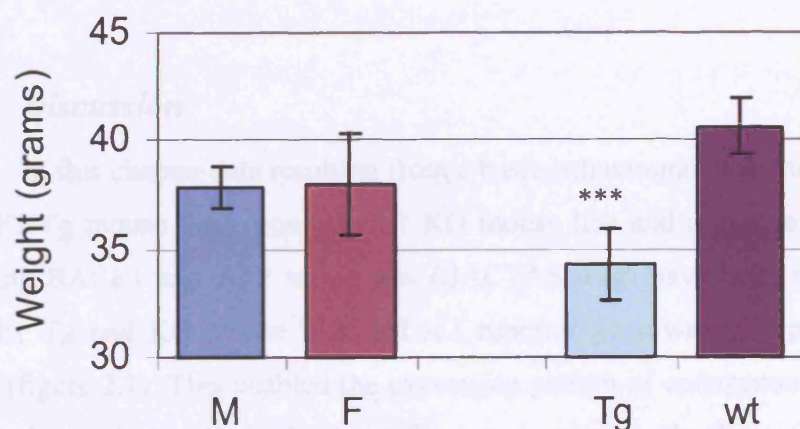
**Figure 2.17 Aggression of BACTAS mice**

Key:  Median  25%-75%  Min-Max. \*\*  $p < 0.01$

Analysis of the recorded incidences of spontaneous aggression in revealed no significant effect of gender,  $p = 0.77$  but a significant effect of genotype,  $p < 0.002$  (Figure 2.17) with BACTAS dTg mice having a median score of 0 with an interquartile range of (0-1) and wt littermate control mice a median score of 1 with an interquartile range of (1-1).

#### 2.3.5.4 Body weight and length

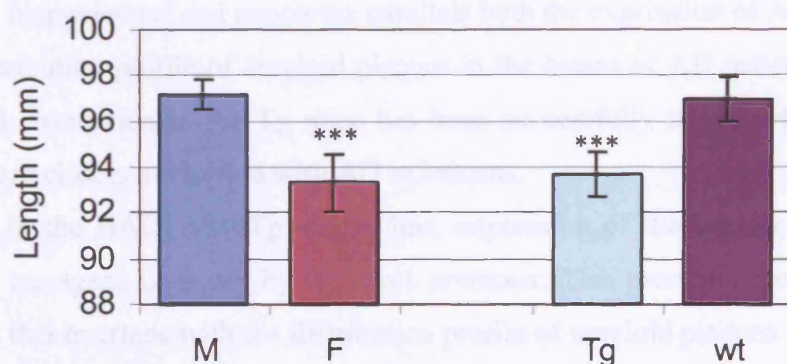
BACTAS dTg mice were lighter and shorter than wt controls. Interestingly no overall difference in weight was seen between male and female mice though female BACTAS dTg mice were lighter than female wt controls. No difference was seen in the body length of female BACTAS dTg mice compared to female controls.



**Figure 2.18 Weight of BACTAS mice**

Mean  $\pm$  SEM \*\*\*  $p < 0.001$

Analysis of body weight revealed no significant effect of gender  $F(1,31) = 0.83$ ,  $p = 0.37$  but a significant effect of genotype  $F(1,31) = 13.3$ ,  $p = 0.001$  with the Tg mice weighing 15.7% less than wt controls (Figure 2.18). An interaction between gender and genotype was observed  $F(1,31) = 5.45$   $p = 0.026$  and in a planned comparison of body weight a significant effect of genotype was seen in the female mice  $F(1,31) = 14.9$ ,  $p = 0.001$  but not the male mice  $F(1,31) = 1.08$ ,  $p = 0.31$ . Female BACTAS dTg mice were 29% lighter than female wt control mice.



**Figure 2.19 Body Length of BACTAS mice**

Mean  $\pm$  SEM \*\*  $p < 0.01$

Analysis of body length revealed a significant effect of gender  $F(1,31) = 15.7$ ,  $p < 0.001$  and genotype  $F(1,31) = 12.6$ ,  $p = 0.001$  with the BACTAS dTg mice measuring an average 3.3mm (3.4%) shorter than wt controls and female mice measuring 3.9mm (4.1%) shorter than male mice (Figure 2.19). There was no interaction between gender and genotype  $F(1,31) = 0.77$ ,  $p = 0.39$ .

## 2.4 Discussion

In this chapter data resulting from a basic behavioural characterisation of two BACE1 Tg mouse lines, one BACE1 KO mouse line and a double Tg mouse line carrying BACE1 and APP transgenes (BACTAS dTg) have been reported. In the BACE1 Tg and KO mouse lines a LacZ reporter gene was incorporated into the locus (figure 2.1). This enabled the expression pattern of endogenous BACE1 to be assessed and hence the selection of mice expressing the human BACE1-LacZ transgene in an expression pattern similar to that driven by the endogenous murine BACE1 promoter. This strategy allowed the comparison of the phenotype of these animals in the knowledge that transgene expression reflected endogenous murine BACE1 gene tissue distribution and relative levels of expression. The possibility that the phenotypic changes reported here are related to the expression of the LacZ reporter gene can be discounted since both the KO and Tg mouse lines express LacZ yet have contrasting phenotypes. Furthermore, other mouse lines expressing the LacZ ubiquitously through endogenous murine promoters do not show similar phenotypes to those reported here (284;285). The high levels of transgene expression seen in hippocampus and neocortex parallels both the expression of APP in mice and the distribution profile of amyloid plaques in the brains of AD patients (139). Thus BACE1 expression in the Tg mice has been successfully targeted to those tissues which are closely associated with AD in humans.

In the BACTAS dTg mouse line, expression of the human APP<sub>KM670/671NL</sub> mutant transgene is driven by the Thy1 promoter. This promoter has an expression pattern that overlaps with the distribution profile of amyloid plaques in the brains of AD patients. The combination of the Thy1 promoter and calmodulin kinase 2 promoter used in the BACTAS dTg mouse model allowed expression of human BACE1 and APP proteins in the same mouse brain regions as those that develop

plaques in human AD patients. The TASTPM mouse line previously published has both Presenilin 1 (PS1<sub>M146V</sub>) and APP<sub>KM670/671NL</sub> transgenes under control of the Thy1 promoter. The expression pattern of these transgenes leads to the generation of amyloid plaques the brains of these mice that parallels the distribution profile of amyloid plaques seen in AD patients (276).

The Tg mouse line overexpressing BACE1 and the KO mouse line lacking BACE1 were on different background strains of mice ([C57Bl/6xCBA] hybrid and [C57Bl/6x129Ola/Sv] hybrid respectively). Thus a direct comparison between these two lines of mice may be confused by differences in background strain. To eliminate this possibility in each analysis the genetically modified line was compared with the corresponding wt littermate mice as control animals. As littermate animals are of the identical respective background strain the effects seen are due to the genetic manipulations alone. The BACTAS double Tg mouse line was on a congenic C57Bl/6J mouse background strain and was also compared to wt littermate control mice. The BACE1 mouse lines were tested in the SHIRPA screen at sexual maturity and the BACTAS mouse line was tested at a time point when a considerable amount of amyloid plaque deposition was expected to be present as seen in TAS10 single Tg mice (275).

Behavioural characterisation of these animals using the battery of tests SHIRPA screen revealed that both BACE1 KO and BACE1 Tg mice were generally normal with most measures unchanged when compared to wt controls. Five of the thirty nine primary SHIRPA test, and one secondary SHIRPA test were significantly different from wt controls. One of the primary SHIRPA differences related to body weight, one to arena activity and two related to limb function. The slightly lower body weight and reduced activity in the test arena of the BACE1 Tg mice plus the slightly firmer limb tone and improved behaviour of the BACE1 KO mice in the wire manoeuvre test did not affect overall locomotion of either line. These behavioural differences did not stem from any differences in background strain since the behaviour of controls in each case was clearly distinguishable from, and intermediate to, the behaviour observed in the genetically modified animals. Interestingly the BACE1 Tg mouse line with a low expression of reporter protein (Line 17) did not have any behaviour that was significantly different from its respective wt controls in any of the primary and secondary SHIRPA measures recorded. Indeed the mouse line represented an intermediate phenotype for many of the significantly altered

behaviours between the high expressing Tg line and the KO mouse line. Increased faecal production, seen in the KO mice, has been reported to correlate with increased emotion and anxiety (286). Furthermore, the decreased exploratory activity of the BACE1 KO mice in the holeboard test is also suggestive of an anxious behavioural phenotype. In contrast to these findings, the Tg BACE1 mice displayed a less anxious behaviour in the holeboard test with significantly more exploration. This behaviour is typical of that seen following administration of anxiolytic drugs to wt mice (287). Overall, the BACE1 KO and higher expressing BACE1 Tg mice present broadly opposite phenotypes with respect to faecal pellet number and holeboard activity. These results indicate a bold, more exploratory behaviour in the BACE1 Tg animals with a higher level of BACE1 expression and a timid, less exploratory behaviour in the BACE1 KO animals compared to controls.

The BACTAS dTg mice were lighter than wt controls overall and a gender specific difference in weight was seen with dTg females being 29% lighter than wt females. Interestingly the dTg mice were shorter than wt control mice and females were shorter than males though this did not have a significant effect on their weight. The increased arena activity and tremor shown by the BACTAS dTg mice is indicative of a poor state of health compared to wt control mice. This may also be reflected in the reduced likelihood of biting when provoked and the reduced aggression shown by the BACTAS mice. These mice were well advanced in plaque pathology and it is possible that they had started to exhibit some of the phenotypes of increased locomotion (288) and passivity (289) sometimes seen in AD patients.

These data indicate altered levels of anxiety in BACE1 KO and Tg mice, hence the behaviour of these mice was investigated in tests sensitive to altered anxiety. In addition, as BACE1 is a key enzyme in the development of neuropathological lesions that contribute to cognitive decline in AD, the response of these lines of mice was investigated in tests sensitive to impaired cognition. The results of these investigations are described in chapter 3.

*Chapter 3 - BACE1 knockout  
mice are anxious and forgetful*



### **3.1 Introduction**

The measurements taken during the basic characterisation of these mice were all related to physical parameters such as weight, responses to physical stimuli such as the hotplate response, or measurements of basic locomotor function such as the Rotarod test. The organ affected in Alzheimer's Disease is the brain, in particular the higher level brain functions. This chapter deals with behaviours that include elements of higher level CNS processing. These are tests of anxiety and cognition.

#### **3.1.1 Anxiety**

Anxiety is an important component of the behaviour of individuals with memory deficits and symptoms of anxiety are commonly exhibited by AD patients (290;291). Similarly, altered anxiety can influence the results of cognition in rodents. During the basic characterisation of the BACE1 mouse models a number of results indicated altered anxiety. These data suggested that formal testing of anxiety in controlled conditions would provide relevant, useful information about the phenotype of these mice.

BACE1 transgenic (Tg) and knockout (KO) mouse lines were examined for anxiety in the elevated plus maze and the open field Test. These comprise part of the tertiary SHIRPA screen.

The open field test is a non conflict model of emotionality and anxiety and was originally described as a test for emotionality in rats (292). Each mouse is placed in the centre of a circular arena and the movement recorded using a video tracking system. Emotionality is determined by level of activity and the proportion of time each mouse spends in the aversive inner zone of the arena. The Elevated Plus maze is a non-conflict animal model of anxiety, which reflects anxiety through the manipulation of natural fear and exploratory drive upon exposure to novel stimuli (293). The elevated plus maze consists of an arena with two enclosed arms and two open arms. Movement is recorded via a video tracking system. The relative movement on open and closed arms is recorded as a measure of anxiety. Both tests are popular procedures in animal psychology (294).

### 3.1.2 Cognition

The formation and storage of memory is poorly understood however a number of key events and experiments have revealed the central role of the hippocampus in the processes underlying memory formation and retention. The first of these arose from an accident when a 27 year old Connecticut epilepsy patient, H.M., underwent surgery in 1953 to cure his affliction. During surgery a mistake with the surgeon's knife led to complete bisection of H.M.'s hippocampus. After recovery from the surgery H.M. found that while his epilepsy had been improved he had also developed temporal retrograde amnesia (295). His condition was not dissimilar to that found in Alzheimer patients and this unhappy accident implicated a role for the hippocampus in memory. In a recent paper Reidel *et al* reported on the results of an experiment where rats were trained to escape a water filled maze over a short period of time. These rats then had their hippocampus inactivated and their memory of the escape was retested. Those rats with an inactive hippocampus behaved as though they had never seen the water filled maze (296). More recently still Day and Morris trained rats repeatedly to find a food reward in a sand maze. After an extended period of training the hippocampus of each rat was inactivated and its memory tested. These rats had an intact memory of the location of the food reward even without an intact hippocampus (297). These experiments support the hypothesis that the hippocampus is required for short-term memory formation but that once formed, this memory is consolidated and stored in another location, currently thought to be the pre-frontal cortex. These experiments also support the observations of memory decline seen in AD patients.

The BACE1 mouse models have changes in the levels of BACE1, an enzyme involved in the processing of APP and known to be involved in the formation of  $\beta$ -amyloid peptides that are the major component of the amyloid plaques found in the brains of Alzheimer's Disease patients (265). As AD patients have changes in learning and memory performance it was prudent to measure these abilities in the BACE1 mouse models. Data from the primary SHIRPA screen indicated altered anxiety in the BACE1 mice and so they were tested for cognition in tests that include different levels of stress. Animals were first examined in the object recognition test as this had the lowest level of stress and were subsequently examined in the fear conditioning test as this has a higher stress component. The object recognition (298) test is dependent on intact peri-rhinal and post-rhinal cortices (299) whereas the



neuronal substrates involved in fear conditioning are amygdala (cued memory) and hippocampus (contextual memory) (300). In addition the double Tg mouse models carrying BACE1 or PS1 with APP transgenes were assessed in the Morris watermaze test adapted for mice in order to examine their spatial working memory (301).

## **3.2 *Materials and Methods***

### **3.2.1 *Animals***

The BACE1 single (Tg) and double transgenic (dTg) mouse models described in this chapter were generated as described in chapter 2. The BACE1 KO and control mice were generated from independent breeding programmes derived from identified homozygous KO and wt littermate control mice then interbreeding homozygotes together and wt littermates together to establish 2 lines of mice with almost identical genetic background strains but not true littermates (i.e. KO and wt mice from the same single generation of breeding).

Anxiety testing was carried out on the same BACE1 KO and Tg mice described in chapter 2 at an age of approximately 4 months and with an N = 10 for each sex and genotype. BACE1 KO and Tg mice from a subsequent breeding effort were tested at an age when differences in cognition have been recorded in mouse models of amyloid deposition (302-304). The object recognition tests were carried out when the BACE1 mice were aged approximately 8 - 9 (KO) and 11 - 12 (Tg) months and the fear conditioning tests were carried out when the mice were aged approximately 12 - 13 (KO) and 15 - 16 (Tg) months of age. For both tests, N = 9 male, 9 female KO; N = 12 male, 11 female wt controls and N = 11 male, 12 female Tg; N = 13 male, 13 female wt controls.

The Amyloid Precursor Protein (APP) is cleaved by 3 enzymes,  $\gamma$ -,  $\alpha$ - and  $\beta$ -secretase. Cleavage by  $\gamma$ - and  $\beta$ -secretases lead to the production of  $\beta$ -amyloid and the rate limiting step in this process is thought to be  $\beta$ -secretase cleavage of APP (90). In order to compare the effects of increasing  $\beta$ -amyloid production with these two enzymes dTg lines that over-express  $\beta$ -secretase with APP (BACTAS) or a component of the  $\gamma$ -secretase complex (Presenilin 1) with APP (TASTPM) were generated by interbreeding the parental mouse strains. The watermaze tests were carried out when the BACTAS and TASTPM dTg mice and wt controls were

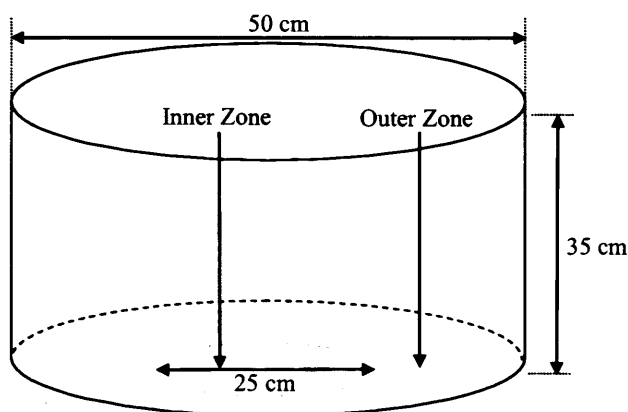
approximately 9-10 months of age. For BACTAS dTg mice N = 8 male, 7 female dTg; N = 8 male, 7 female wt and for TASTPM dTg mice, N = 7 male, 8 female dTg; N = 7 male, 8 female wt.

### 3.2.2 Anxiety

Anxiety was tested using the open field test of emotionality and the elevated plus maze test of anxiety. Each of these tests relies on the innate behaviours of the mouse. The natural inquisitive nature of the mouse is balanced by a natural fear of open, exposed spaces.

#### 3.2.2.1 Open field test of emotionality

This test required the mouse to be placed in the centre of a circular arena 50 cm in diameter with walls 35 cm high. The arena was made of white Perspex and lit from above with white light. This created an environment that can be divided into



two zones, a more aversive inner zone, where the mouse is most fearful, and a less aversive outer zone where the mouse can walk near the wall of the arena. Each mouse was placed in the centre of the arena and allowed to explore for 10 minutes. During this time the movement of the

mice was monitored by a video camera and recorded by a video cassette recorder. The camera was linked to a personal computer and all of the movement tracked and analysed using a video tracking system. In between each animal the arena was cleaned with 70% ethanol.

### 3.2.2.2 Elevated plus maze test of anxiety

The test used an arena made from black Perspex in the shape of a plus. This had arms that are 30 cm long and 5 cm wide. Two opposing arms were enclosed with



walls 15 cm high and the other two opposing arms were left open. The four arms were raised 50 cm above the floor and illuminated with red light. Each animal was left to explore the maze for 5 minutes. During this test the movement of the mice was monitored by a video camera and recorded by a video cassette recorder. The camera was linked to a personal computer and all of the movement tracked

and analysed using video tracking software. In between each animal the arena was cleaned with 70% alcohol.

### 3.2.3 Cognition

The Morris watermaze, object recognition and fear conditioning test were used to test cognition in the different mouse models. These tests differ in execution but, depending on the protocol used, they examine the function of the hippocampus, rhinal cortices and amygdala.

#### 3.2.3.1 Object recognition

The object recognition test utilises the natural propensity of rodents to investigate unfamiliar objects. Mice were allowed to habituate to a plain black



Perspex circular arena, 50 cm in diameter with 35 cm high walls, with no objects in it for 30 minutes on day 1. The arena was cleaned with 70% alcohol between each habituation. The following day the mice were placed, one at a time, into the arena and allowed to explore a white, Perspex cylinder, 2.5 cm in height and 2.5 cm in diameter, designated as the familiar object, for 10 minutes. 3 hours later the mice were placed into the same arena for 10

minutes and exposed to the familiar white cylinder and a novel object, a black Perspex cube with 1 cm sides. Object investigation was deemed to be when the head

of the mouse was pointing at the object and the nose was less than 1 cm from the object. The time spent investigating each object was recorded and an index of novelty preference calculated. Mice that remembered the familiar object after a delay of 3 hours were considered to have an unimpaired memory spending significantly more time investigating the novel object than the familiar object.

### 3.2.3.2 Fear conditioning

Mice were exposed to a fear conditioning test chamber (TSE Systems GmbH, Germany) approximately 19 cm x 35 cm x 20 cm in height, width and depth. This chamber consisted of a clear Perspex box with 4 walls and a lid. The floor of the chamber comprised metal bars, 4 mm in diameter and 8.9 mm apart. Surrounding the



box were two levels of motion sensors that recorded the horizontal and vertical movement of the mice. In addition a sound source and light source were on

the outside of the chamber but inside a wooden box that surrounded the entire unit. The metal grid, motion sensors, light and sound sources were all connected to a control box which was connected to a PC. The sequence of sound, light, shock and pauses were controlled by software on the PC. Using a combination of the 4 parameters; shock, light, sound and pause; the mice were trained to associate an audible cue with the conditioned stimulus of a foot shock. Subsequently their response to the unconditioned stimulus of the housing context and conditioned stimulus of auditory cue were measured.

The test protocol for the mice began with a 3 minute habituation to the chamber, followed by a 30 second delay and then a 30 second period of an audible tone ending in a 2 second, 0.7 mA electric shock to the feet delivered through metal grids in the floor of the chamber. This tone & shock pairing was repeated a second time. 24 hours later the mice were returned to the chamber for 3 minutes, removed while the chamber was cleaned with 70% methanol and context changed (by the addition of a paper floor covering, a diagonal divider inserted into the chamber and brightening the light) then returned to the chamber in its new context for 3 minutes.



Following this period the tone was repeated for 30 seconds. The movement of the mice was tracked using infra-red beams inside the chamber and analysed by a computer.

### 3.2.3.3 Watermaze

This test was designed for rats but has been adapted for use in mice and relies on the mouse's ability to swim combined with its drive to escape the water. The maze was 100 cm in diameter by 30 cm deep and was arbitrarily orientated to North, South, East and West directions. A white partitioning board placed to the West to obscure the contents of the rest of the room. Three orientation cues were used in the



test. A red room door with a backed out window to the North, a large black cross made from a black laminated A4 sheet of paper to the West on the white partition wall and a hollow square again made from a black laminated A4 sheet of paper was to East. Four 8-inch wide black horizontal stripes were added to the shower curtain to South. An

additional wall designed to prevent mice jumping from the maze once they had reached the platform was constructed from laminated sheets of A4 paper. The maze was filled with 120 litres of tap water made opaque by the addition of latex solution. The temperature of the maze was  $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$  controlled by the addition of hot tap water.

The watermaze test consisted of an initial phase of visual cue training over 3 days with 1 session per day and 4 trials per session. This phase of the test was designed to examine the visual acuity and swimming ability of the animals as well as to habituate them to the pool and train them to escape the water. Training trials were massed where each trial immediately followed the preceding trial with an inter-trial interval of approximately 90 seconds. After each session mice were dried with a towel and returned to their home-cage. The visual cues in the room were excluded with a white curtain and the mice placed in the watermaze and trained to swim to a visual cue attached to the platform during each trial. After a 2 day delay the

orientation cues to North, South, East and West were revealed and the mice trained to escape the water to a hidden platform over 5 days of visual cue training with 1 session per day and 4 trials per session. During spatial cue training mice were allowed up to 60 seconds to find the platform after which time they were guided to the platform by hand. On reaching the platform mice were left on it for 20 seconds before removal. Seven days after spatial cue training the memory of the mice was tested in a single probe trial. During the probe trial the mice were allowed to swim in the pool for 60 seconds with no platform present. After 60 seconds the mouse was rescued, dried and returned to its home-cage. After this probe trial mice were subjected to one of two further tests. They were either tested for changes in synaptic plasticity with one session of re-training followed by 5 trials of reversal training where the target platform was reversed and the ability of the mice to learn the new platform position tested, or they were tested for extended memory retrieval with a second probe trial 7 days after the first.

During the watermaze tests the mice were tracked using a closed circuit TV system connected to a VCR and PC. The movement of the mice was analysed using software designed for tracking animal movement in the watermaze called 'Water 2020' ([www.hvsimage.com](http://www.hvsimage.com)).

Statistical analyses were conducted using Statistica<sup>TM</sup> (Statsoft, Inc). ANOVA was performed to compare the effects of genotype on each of the behavioural variables. The differences between individual means were assessed with post hoc simultaneous comparisons using the Fisher LSD test.

### **3.3 Results**

#### **3.3.1 Anxiety**

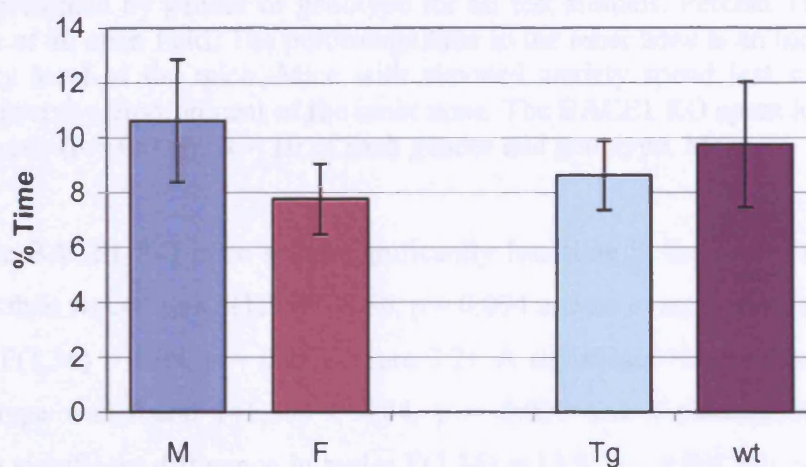
A number of significant differences were found in the behaviour of the mice when tested in the open field test of emotionality and elevated plus test of anxiety.

BACE1 Tg mice did not differ from controls in open field activity. Overall BACE1 KO mice spent 48.1% less time and travelling 39.2% less distance in the inner zone of an open field indicating an anxious phenotype however this behaviour was attributable to male and not female KO mice when compared to the same sex wt controls. BACE1 KO mice also covered a greater distance than wt controls though this measure failed to reach statistical significance. BACE1 Tg mice spent 26% more

time and BACE1 KO mice 16% less time on the open arms of an elevated plus maze than controls though these measures failed to reach significance. BACE1 Tg also demonstrated significantly fewer stretch attend behaviours than controls while in the maze and BACE1 KO mice groomed themselves twice as much as control mice. These results suggest opposing phenotypes with BACE1 Tg mice less anxious and BACE1 KO mice more anxious than respective controls.

### 3.3.1.1 Open field test of emotionality

No statistically significant effect of gender or genotype was seen on any measure when the Tg mice were tested in an open field, however the KO mice demonstrated an anxious phenotype.

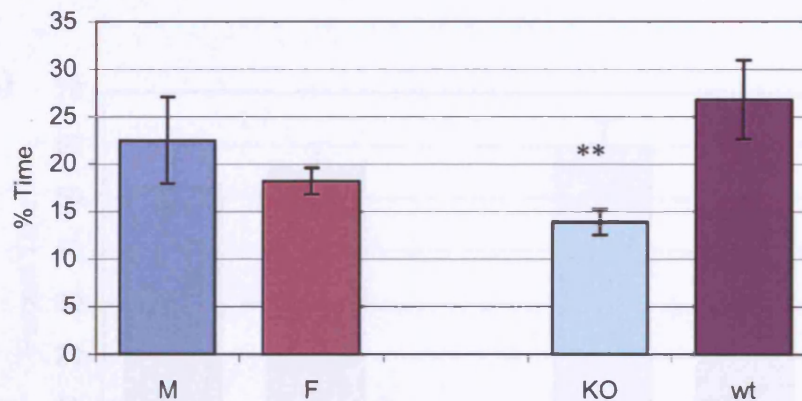


**Figure 3.1 Percent Time in inner zone for BACE1 Tg mice**

Data are presented by gender or genotype for all test animals. Percent Time in the inner zone of an open field. The percentage time in the inner zone is an indication of the anxiety level of the mice. Mice with elevated anxiety spend less time in the relatively aversive environment of the inner zone. The BACE1 Tg spent less time in the inner zone though this measure was not significant ( $p = 0.67$ ).  $N = 10$  of each sex and genotype. Mean  $\pm$  SEM

No significant effect of gender or genotype was seen for the percent time travelled in the inner zone of the open field (Figure 3.1) [gender  $F(1,36) = 1.16$ ,  $p = 0.29$ ; genotype  $F(1,36) = 0.17$ ,  $p = 0.67$ ]. Neither was any effect of gender or genotype seen for overall distance travelled in the arena [gender  $F(1,36) = 0.62$ ,  $p = 0.43$ ; genotype  $F(1,36) = 0.01$ ,  $p = 0.92$ ]. No gender x genotype interaction was seen for any of these measures.



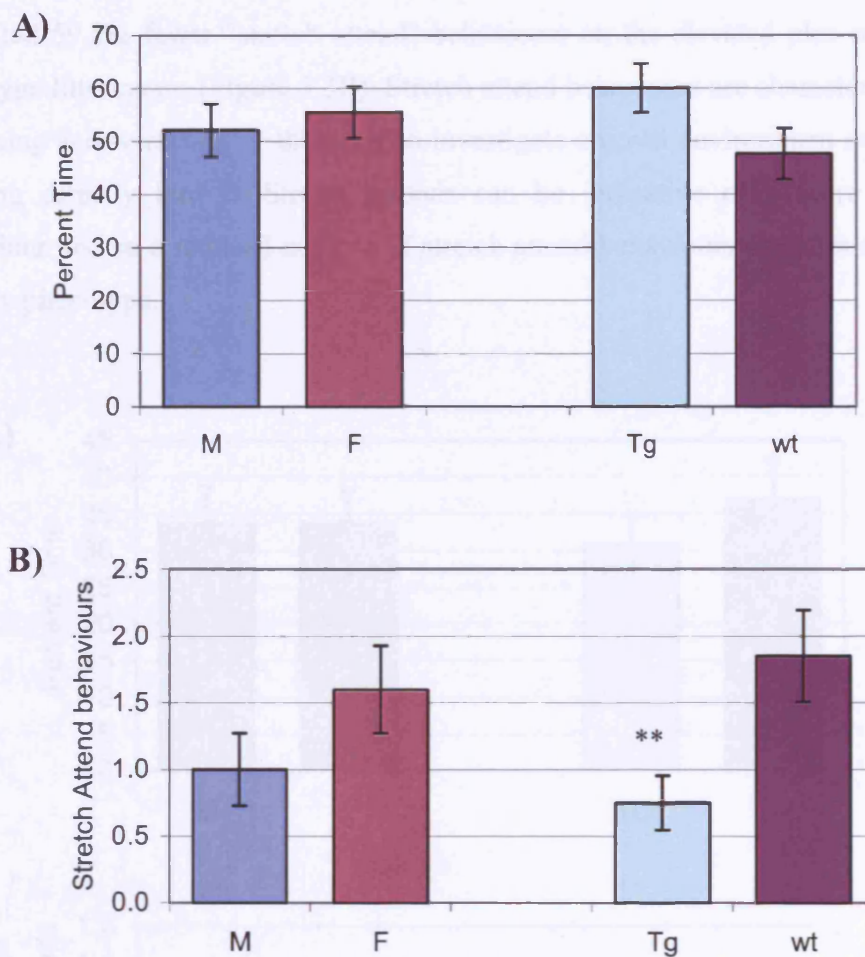


**Figure 3.2 Percent Time in inner zone for BACE1 KO mice**

Data are presented by gender or genotype for all test animals. Percent Time in the inner zone of an open field. The percentage time in the inner zone is an indication of the anxiety level of the mice. Mice with elevated anxiety spend less time in the relatively aversive environment of the inner zone. The BACE1 KO spent less time in the inner zone ( $p = 0.004$ ).  $N = 10$  of each gender and genotype. Mean  $\pm$  SEM. \*\* $p < 0.01$

The BACE1 KO mice spent significantly less time in the inner zone of the open field than wt controls  $F(1,36) = 9.56$ ,  $p = 0.004$  and no overall effect of gender was seen  $F(1,36) = 1.08$ ,  $p = 0.31$  (Figure 3.2). A significant interaction of gender and genotype was found  $F(1,36) = 4.84$ ,  $p = 0.038$  and a planned comparison revealed a significant difference in males  $F(1,36) = 13.8$ ,  $p = 0.001$  but not females  $F(1,36) = 0.44$ ,  $p = 0.55$ . No significant effect of gender  $F(1,36) = 0.08$ ,  $p = 0.782$  or genotype  $F(1,36) = 3.31$ ,  $p = 0.07$  on the distance travelled in the arena was seen and no gender  $\times$  genotype interaction was seen for this measure. Overall the male BACE1 KO mice covered a similar distance to wt controls and yet spent 65% less time and travelled 58% less distance in the inner zone of an open field. These data indicate a sex specific anxiety effect of removing endogenous BACE1 expression in these mice.

### 3.3.1.2 Elevated plus maze test of anxiety



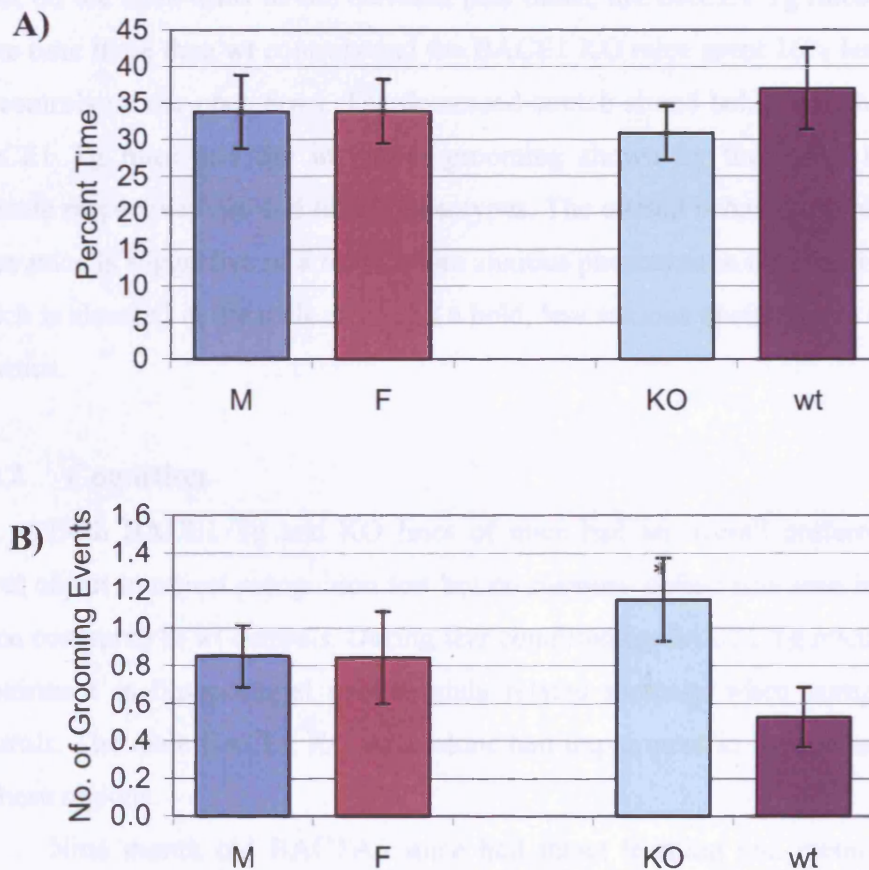
**Figure 3.3 Elevated Plus maze test of anxiety for BACE1 Tg mice**

Data are presented by gender or genotype for all test animals. **A).** Percent Time on the open arms. The percentage time on the open arms of an elevated plus maze is an indication of the anxiety level of the mice. Mice with elevated anxiety spend less time in the relatively aversive environment of the open arms. The BACE1 Tg spent more time on the open arms indicating reduced anxiety though this measure failed to reach significance. ( $p = 0.077$ ). **B).** Stretch Attend behaviours. Tg mice exhibited fewer stretch attend behaviours ( $p = 0.009$ ) indicating reduced anxiety.  $N = 10$  for each gender and genotype. Mean  $\pm$  SEM. \*\* $p < 0.01$

The percent time spent in the Open Arms of the elevated plus maze was analysed and no significant effect of gender  $F(1,36) = 0.23$ ,  $p = 0.63$  or genotype  $F(1,36) = 3.32$ ,  $p = 0.077$  was found for the BACE1 Tg mice (Figure 3.3A). One significant difference was found for these mice when the number of "stretch attend"



behaviours was analysed a significant effect of genotype  $F(1,36) = 7.69$ ,  $p = 0.009$  but not gender  $F(1,36) = 2.29$ ,  $p = 0.14$  was found. No significant gender x genotype interaction  $F(1,35) = 0.06$ ,  $p = 0.80$  was seen for this measure. The BACE1 Tg mice exhibited 59.5% fewer "stretch attend" behaviours on the elevated plus maze than wild-type littermates (Figure 3.3B). Stretch attend behaviours are characterised by a stretching and retracting of the body to investigate a novel environment rather than walking directly into it. Stretch attends can be indicative of a more cautious behaviour and so a reduced number of stretch attend behaviours suggests a reduced anxiety phenotype.



**Figure 3.4 Elevated Plus maze test of anxiety for BACE1 KO mice**

Data are presented by gender or genotype for all test animals. **A).** Percent Time on the open arms. The percentage time on the open arms of an elevated plus maze is an indication of the anxiety level of the mice. Mice with elevated anxiety spend less time in the relatively aversive environment of the open arms. The BACE1 KO spent less time on the open arms indicating increased anxiety though this measure failed to reach significance. ( $p = 0.38$ ). **B).** Grooming behaviour. KO mice demonstrated more bouts of grooming ( $p = 0.03$ ) indicating increased anxiety.  $N = 10$  for each gender and genotype. Mean  $\pm$  SEM. \* $p < 0.05$

Analysis of the percent time spent in the open arms of the elevated plus maze by the BACE1 KO mice revealed no significant effect of gender  $F(1,35) < 0.001$ ,  $p = 0.99$  or genotype  $F(1,35) = 0.77$ ,  $p = 0.38$  (Figure 3.4A). One significant difference was found for these mice when the number of "grooming" behaviours was examined and a significant effect of genotype  $F(1,35) = 4.84$ ,  $p = 0.03$  but not gender  $F(1,35) = 0.01$ ,  $p = 0.94$  was found. The BACE1 KO mice exhibited over twice as many "grooming" behaviours on the elevated plus maze than wild-type littermates (Figure 3.4B).

While there was no significant difference for either genotype in the time spent on the open arms of the elevated plus maze, the BACE1 Tg mice spent 26% more time there than wt controls and the BACE1 KO mice spent 16% less time than wt controls on the open arms. The decreased stretch attend behaviour shown by the BACE1 Tg mice and the increased grooming shown by the BACE1 KO mice indicate respective bold and timid phenotypes. The overall behavioural phenotype of these mice is suggestive of a timid, more anxious phenotype in the BACE1 KO mice which is elevated in the male mice and a bold, less anxious phenotype in the BACE1 Tg mice.

### 3.3.2 Cognition

Both BACE1 Tg and KO lines of mice had an overall preference for the novel object in object recognition test but no memory deficit was seen in either line when compared to wt controls. During fear conditioning BACE1 Tg mice showed no impairment in hippocampal or amygdala related memory when compared to wt controls. The male BACE1 KO mice alone had impairment in the memory function of these regions.

Nine month old BACTAS mice had intact learning and memory with no difference in synaptic plasticity when compared to wt littermate control mice in the watermaze test. At a similar age, the female, but not male, TASTPM mice had impaired learning and a poor memory of the accurate position of the escape platform in the watermaze.



### 3.3.2.1 Object recognition test

In the object recognition (OR) test the preference for a novel object shown by the BACE1 mice was assessed when the mice were allowed to explore an arena containing an object and subsequently were exposed to the same arena with the now familiar object and a new, novel object. The index of time spent investigating the novel and familiar objects was taken as an indication of their recall of the familiar object and hence their overall memory function.

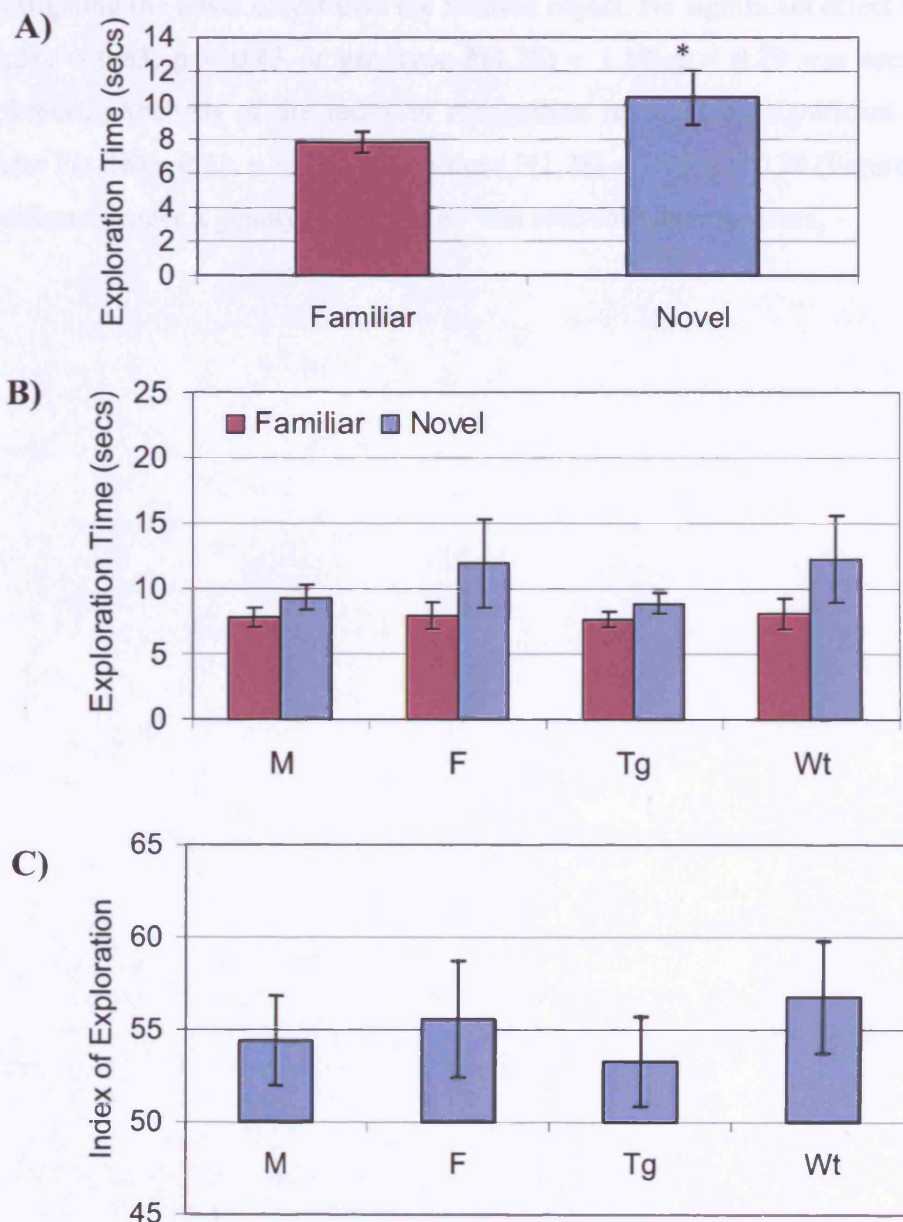
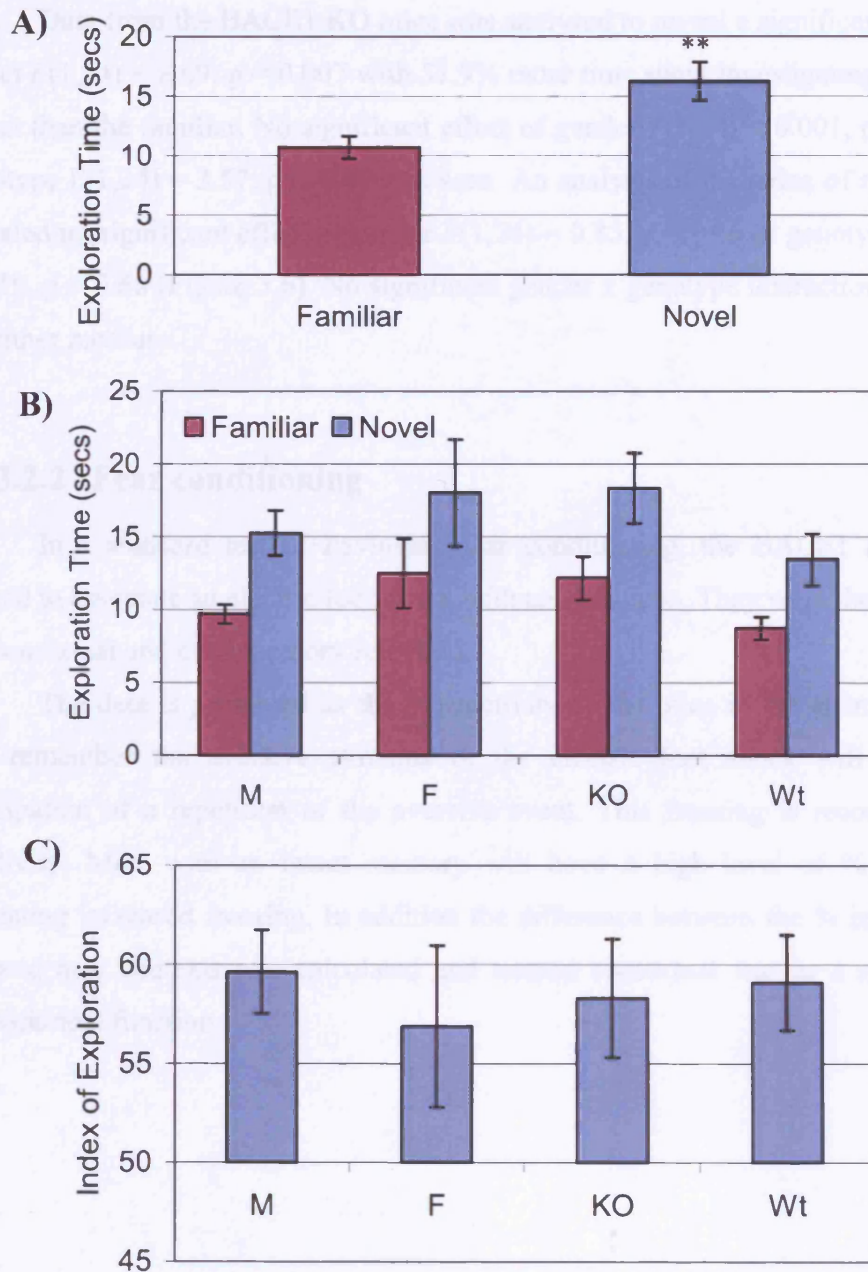


Figure 3.5 Object recognition in Tg mice

**A).** Overall object preference. **B).** Time spent on novel or familiar objects for each group. Unimpaired mice spend more time on the novel object than familiar object. Overall there was no significant difference between Tg and wt mice ( $p = 0.29$ ) indicating memory recall in the KO mice was intact. **C).** Index of recognition as an alternative measure of memory recall. No significant effect of gender or genotype was found. Data are presented by gender or genotype for all test animals.  $N = 11$  M Tg, 12 F Tg, 13 M wt, 13 F wt controls. Mean  $\pm$  SEM.  $*p < 0.05$

Data from the OR test of the BACE1 Tg mice were analysed to reveal a significant effect of object  $F(1,28) = 6.54$ ,  $p = 0.016$  with 33.5% more time spent investigating the novel object than the familiar object. No significant effect of gender  $F(1,28) = 0.63$ ,  $p = 0.43$  or genotype  $F(1,28) = 1.17$ ,  $p = 0.29$  was seen on this preference. Analysis of the index of recognition revealed no significant effect of gender  $F(1,28) = 0.21$ ,  $p = 0.65$  or genotype  $F(1,28) = 1.16$ ,  $p = 0.29$  (Figure 3.5). No significant gender x genotype interaction was seen for either measure.





**Figure 3.6 Object recognition of KO mice**

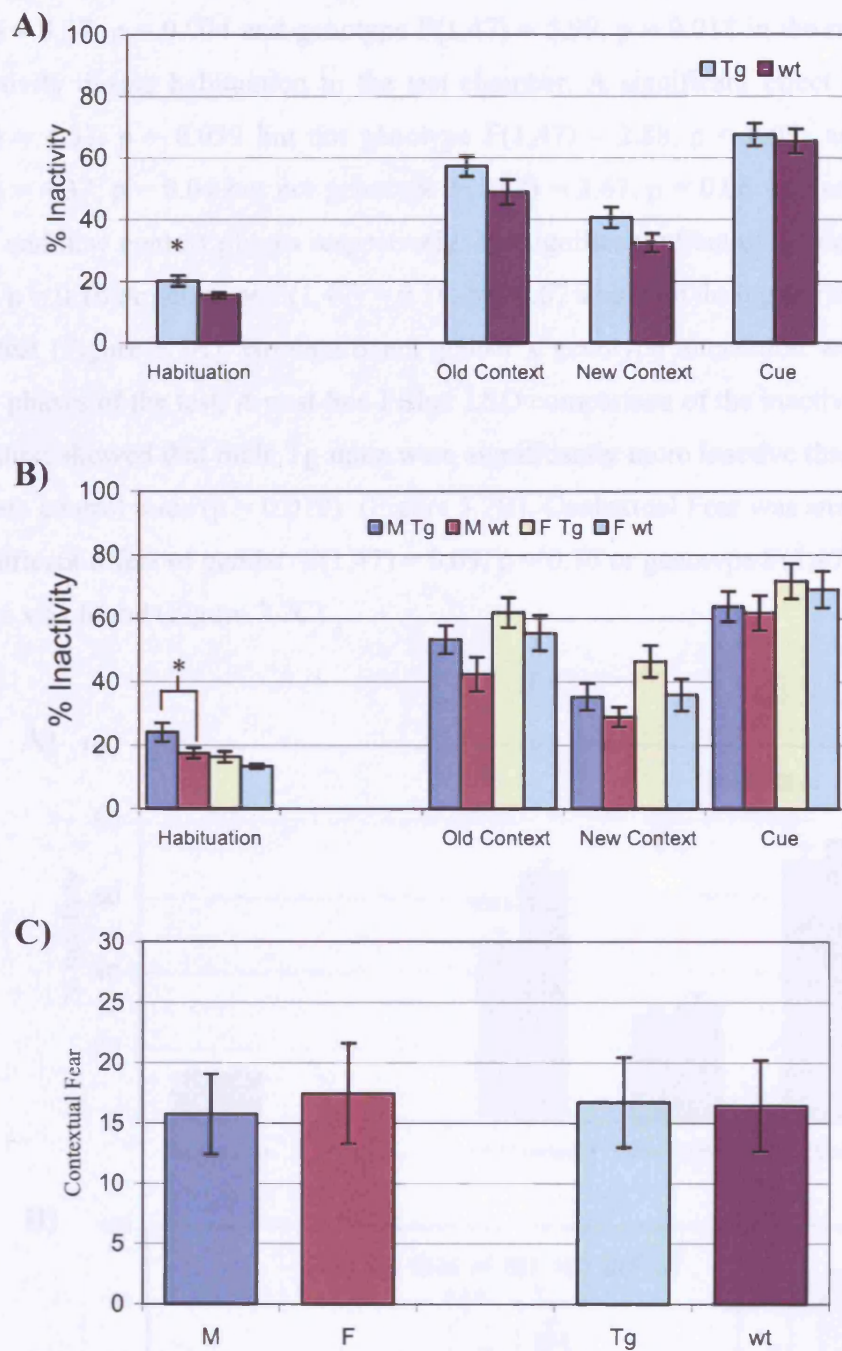
**A).** Overall object preference. **B).** Time spent on novel or familiar objects for each group. Unimpaired mice spend more time on the novel object than familiar object. Overall there was no significant difference between KO and wt mice ( $p = 0.071$ ) indicating memory recall in the OK mice was intact. **C).** Index of recognition as an alternative measure of memory recall. No effect of gender or genotype was found. Data are presented by gender or genotype for all test animals.  $N = 9$  M KO,  $9$  F KO,  $12$  M wt,  $11$  F wt controls. Mean  $\pm$  SEM. \*\*  $p < 0.01$

Data from the BACE1 KO mice was analysed to reveal a significant effect of object  $F(1,24) = 8.69$ ,  $p = 0.007$  with 51.9% more time spent investigating the novel object than the familiar. No significant effect of gender  $F(1,24) < 0.001$ ,  $p = 0.96$  or genotype  $F(1,24) = 3.57$ ,  $p = 0.07$  was seen. An analysis of the index of recognition revealed no significant effect of gender  $F(1,24) = 0.85$ ,  $p = 0.36$  or genotype  $F(1,24) = 0.19$ ,  $p = 0.66$  (Figure 3.6). No significant gender x genotype interaction was seen for either measure.

### **3.3.2.2 Fear conditioning**

In a standard test of Pavlovian fear conditioning, the BACE1 mice were trained to associate an electric foot shock with an audio cue. They were then assessed for contextual and cued memory retention.

The data is presented as the % inactivity of the mice in the chamber. Mice that remember the aversive stimulus of the electric foot shock will freeze in anticipation of a repetition of the aversive event. This freezing is recorded as % inactivity. Mice with an intact memory will have a high level of % inactivity indicating increased freezing. In addition the difference between the % inactivity in old and new contexts was calculated and termed contextual fear as a measure of hippocampal function.

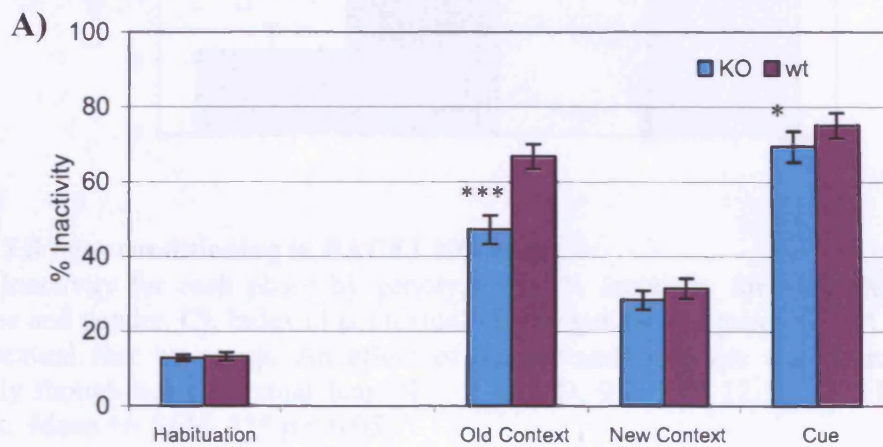


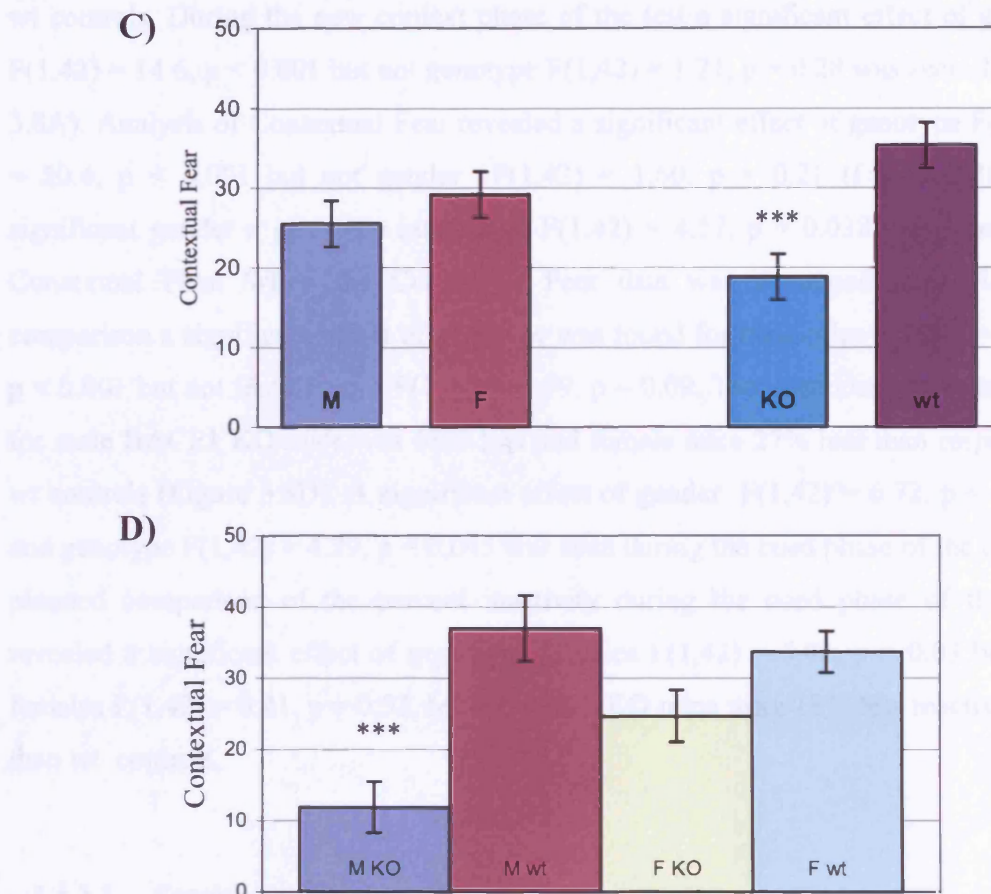
**Figure 3.7 Fear conditioning in Tg mice**

A). % Inactivity for each phase by genotype. B). % Inactivity for each phase by genotype and gender. C). Index of contextual fear. An effect of gender and genotype was seen in % inactivity though not contextual fear. N = 11 M Tg, 12 F Tg, 13 M wt, 13 F wt controls. Mean  $\pm$  SEM. \*  $p < 0.05$



Analysis of the BACE1 Tg mouse data revealed a significant effect of gender  $F(1,47) = 9.37$ ,  $p = 0.004$  and genotype  $F(1,47) = 5.99$ ,  $p = 0.018$  in the measure of % inactivity during habituation to the test chamber. A significant effect of gender  $F(1,47) = 4.51$ ,  $p = 0.039$  but not genotype  $F(1,47) = 2.88$ ,  $p = 0.09$  and gender  $F(1,47) = 4.37$ ,  $p = 0.04$  but not genotype  $F(1,47) = 3.67$ ,  $p = 0.06$  was seen during the old and new context phases respectively. No significant effect of gender  $F(1,47) = 2.03$ ,  $p = 0.16$  or genotype  $F(1,47) = 0.18$ ,  $p = 0.67$  was seen during the cued phase of the test (Figure 3.7A). No significant gender x genotype interaction was seen in these 3 phases of the test. A post-hoc Fisher LSD comparison of the inactivity during habituation showed that male Tg mice were significantly more inactive than male wt littermate control mice ( $p = 0.019$ ) (Figure 3.7B). Contextual Fear was analysed and no significant effect of gender  $F(1,47) = 0.09$ ,  $p = 0.76$  or genotype  $F(1,47) < 0.001$ ,  $p = 0.96$  was found (Figure 3.7C).





**Figure 3.8 Fear conditioning in BACE1 KO mice**

**A).** % Inactivity for each phase by genotype. **B).** % Inactivity for each phase by genotype and gender. **C).** Index of contextual fear by gender and genotype. **D).** Index of contextual fear by group. An effect of gender and genotype was seen in % inactivity though not contextual fear. N = 9 M KO, 9 F KO, 12 M wt, 11 F wt controls. Mean  $\pm$  SEM. \*\*\*  $p < 0.05$

Analysis of the data generated from the BACE1 KO mouse fear conditioning test revealed no significant effect of gender  $F(1,42) = 0.07$ ,  $p = 0.79$  or genotype  $F(1,42) = 0.09$ ,  $p = 0.76$  in the measure of inactivity during habituation to the test chamber. A significant effect of gender  $F(1,42) = 17.1$ ,  $p < 0.001$  and genotype  $F(1,42) = 24.1$ ,  $p < 0.001$  with a near significant interaction  $F(1,42) = 3.84$ ,  $p = 0.057$  was found during the old context phase (Figure 3.8A) where BACE1 KO mice were found to be 29.2% less inactive than wt controls. A planned comparison of the inactivity during the old context phase revealed a significant effect of genotype for male mice  $F(1,42) = 22.5$ ,  $p < 0.001$  and female mice  $F(1,42) = 4.56$ ,  $p = 0.04$ . Male KO mice were 47% less inactive and female mice 17% less inactive than respective

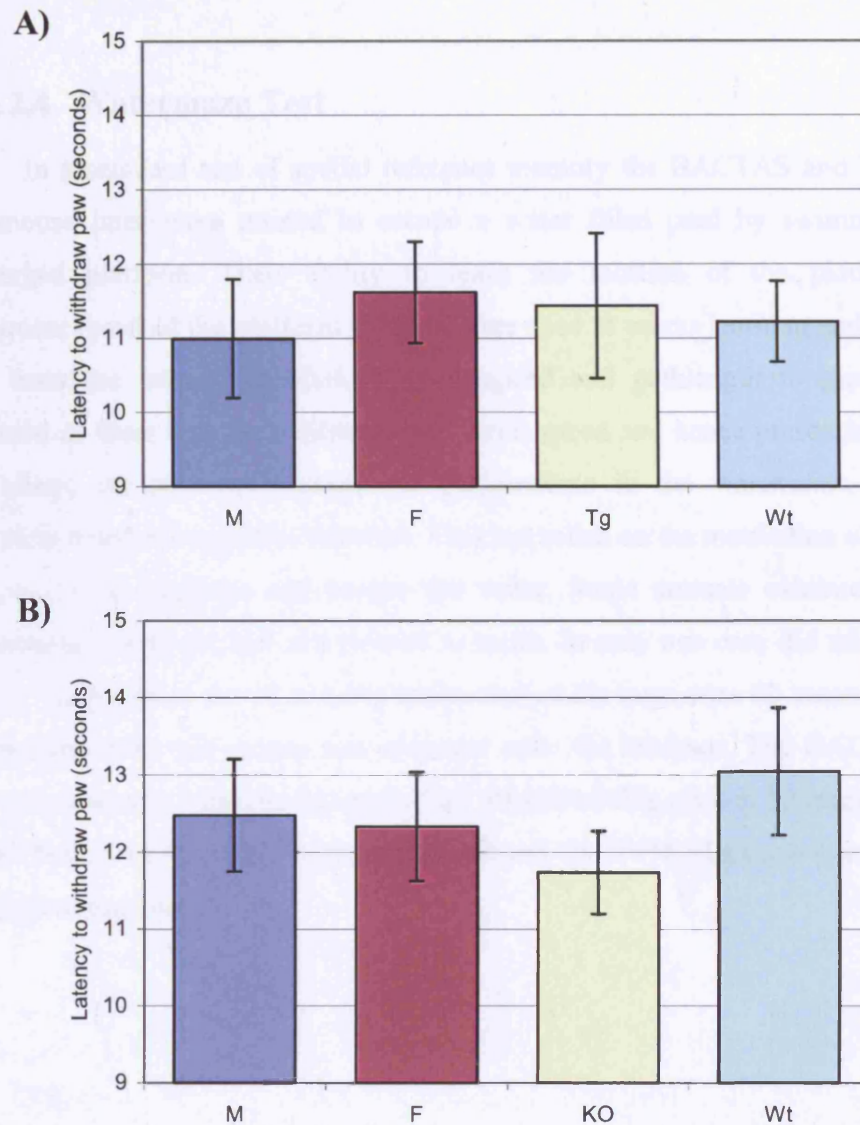


wt controls. During the new context phase of the test a significant effect of gender  $F(1,42) = 14.6$ ,  $p < 0.001$  but not genotype  $F(1,42) = 1.21$ ,  $p = 0.28$  was seen (Figure 3.8A). Analysis of Contextual Fear revealed a significant effect of genotype  $F(1,42) = 20.4$ ,  $p < 0.001$  but not gender  $F(1,42) = 1.60$ ,  $p = 0.21$  (Figure 3.8C). A significant gender x genotype interaction  $F(1,42) = 4.57$ ,  $p = 0.038$  was found for Contextual Fear. When the Contextual Fear data was examined in a planned comparison a significant effect of genotype was found for male mice  $F(1,42) = 21.2$ ,  $p < 0.001$  but not female mice  $F(1,42) = 2.99$ ,  $p = 0.09$ . The contextual fear measure for male BACE1 KO mice was 68% less and female mice 27% less than respective wt controls (Figure 3.8D). A significant effect of gender  $F(1,42) = 6.72$ ,  $p < 0.001$  and genotype  $F(1,42) = 4.29$ ,  $p = 0.045$  was seen during the cued phase of the test. A planned comparison of the percent inactivity during the cued phase of the test revealed a significant effect of genotype in males  $F(1,42) = 5.02$ ,  $p = 0.03$  but not females  $F(1,42) = 0.41$ ,  $p = 0.52$ . Male BACE1 KO mice were 18% less inactive and than wt controls.

### 3.3.2.3 Sensitivity to pain

During the fear conditioning mice were exposed to an electrical shock to the foot. The response of the mouse may be confounded by differences in sensitivity to pain. The regions of the brain thought to be involved in pain perception include the primary and secondary somatosensory cortices and the anterior cingulate cortex (305). Though these lines of mice did not show differences in pain sensitivity when tested during primary SHIRPA, in order to verify the pain sensitivity in these older BACE1 mice they were re-tested for thermal nociception using the Hotplate test. The mice were placed on a metal plate heated to 50°C and the latency for them to withdraw their paw from the heat was recorded. No differences were seen between BACE1 Tg or KO mice and respective wt controls.





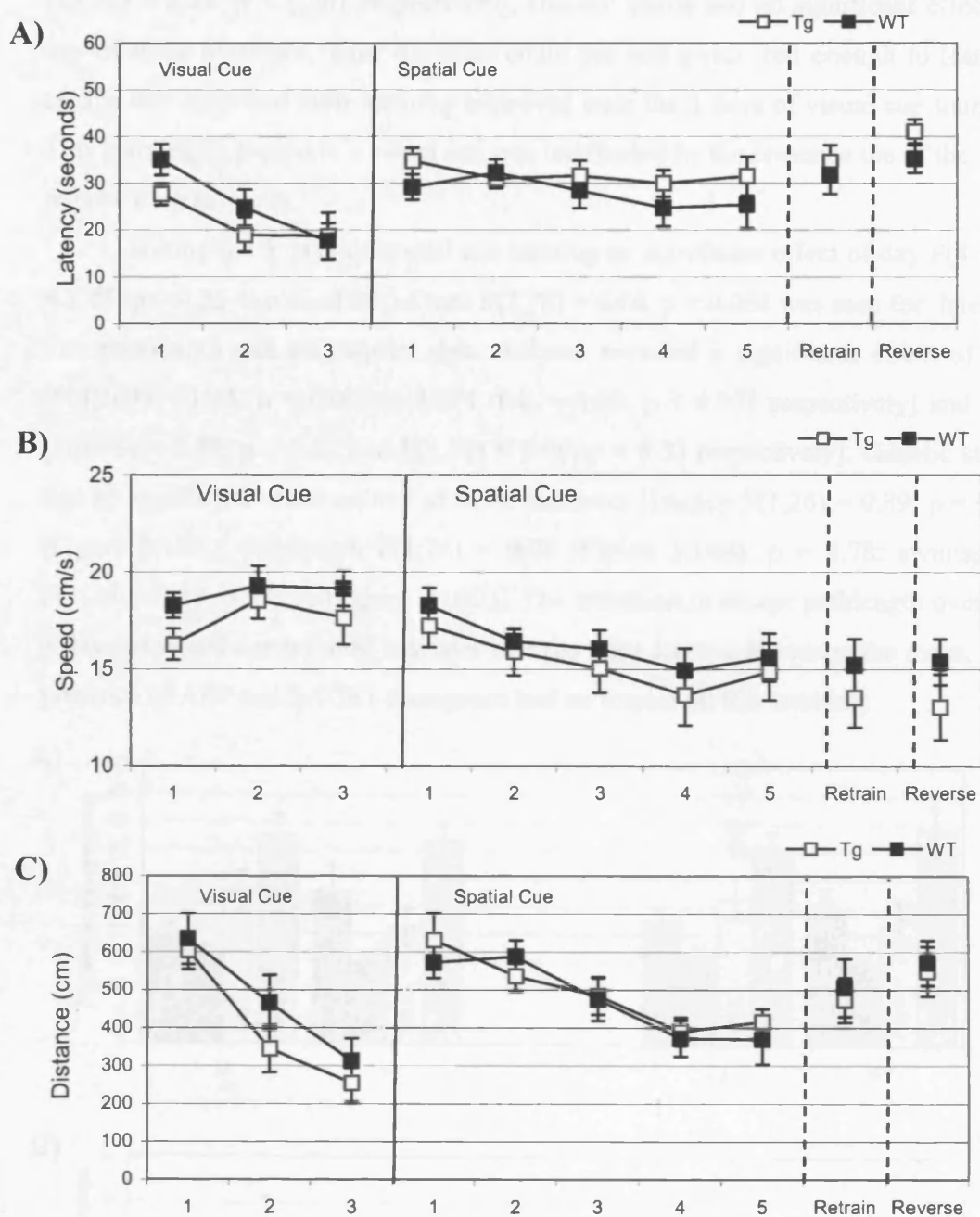
**Figure 3.9 Thermal Nociception in BACE1 mice**

A). Thermal nociception in BACE1 Tg mice. B). Thermal nociception in BACE1 Tg mice. No differences were seen in thermal nociception when compared to wt controls for each line of mice. For A) N = 11 M Tg, 12 F Tg, 13 M wt, 13 F wt controls. For B) N = 9 M KO, 9 F KO, 12 M wt, 11 F wt controls. Mean  $\pm$  SEM

In a test of thermal nociception, the latency to respond to the heat stimulus was recorded for the BACE1 Tg mice (Figure 3.9A). Analysis of this latency revealed no significant effect of gender  $F(1,21) = 0.32$ ,  $p = 0.58$  or genotype  $F(1,21) = 0.03$ ,  $p = 0.87$ . BACE1 KO mice were tested in the same way and no significant effect of gender  $F(1,35) < 0.001$ ,  $p = 0.96$  or genotype  $F(1,35) = 1.64$ ,  $p = 0.21$  was seen in latency to respond (Figure 3.9B). No gender x genotype interaction was seen for the either line of mice.

#### **3.3.2.4 Watermaze Test**

In a standard test of spatial reference memory the BACTAS and TASTPM dTg mouse lines were trained to escape a water filled pool by swimming to a submerged platform. Their ability to learn the location of the platform and subsequent recall of the platform position were used to assess learning and memory. Data from the latency to platform, swimspeed and pathlength to platform are presented as there may be a difference in swim speed and hence presenting latency data alone, the standard measure of performance in the watermaze, may not accurately represent cognitive function. This test relied on the motivation of the mice to swim to the platform and escape the water. Some animals exhibited learned helplessness during the test and refused to swim. In only one case did this occur to such an extent (26 of the 32 training trials reached the maximum 60 second latency) that the data from this mouse was excluded from the analyses. The BACTAS dTg mice were tested at 9 months of age and the TASTPM dTg mice at 10 months of age. Overall BACTAS dTg mice were unimpaired and TASTPM dTg mice were impaired in cognitive function.



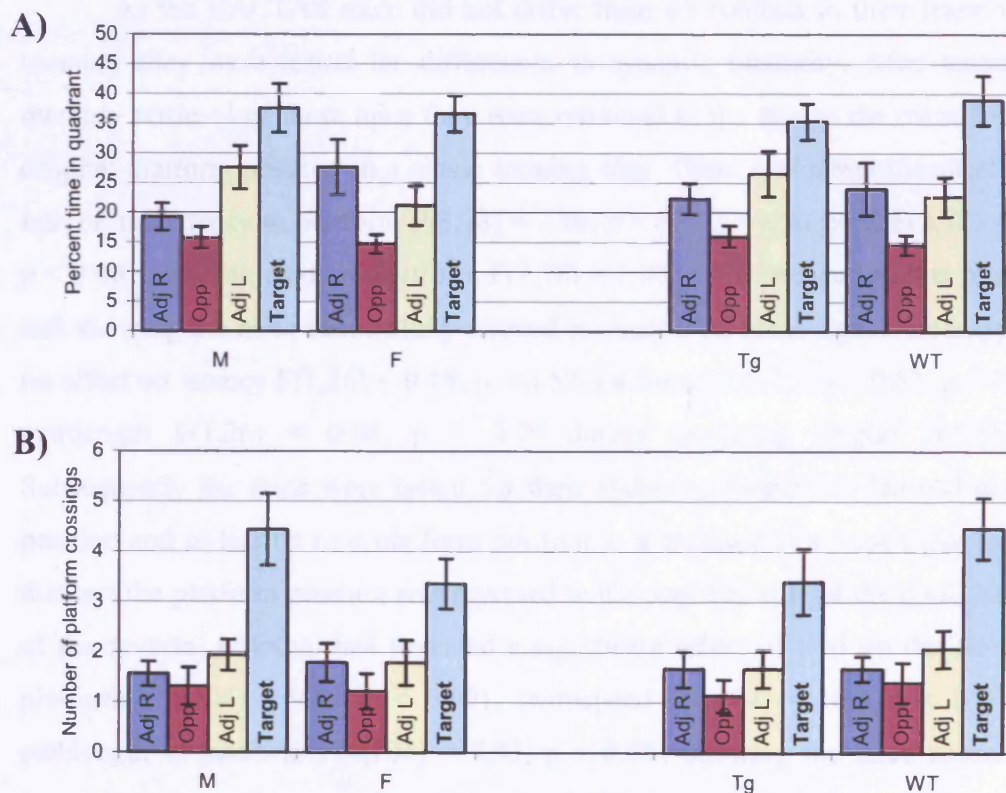
**Figure 3.10 Learning in BACTAS dTg mice during watermaze training**

A). Latency to platform B). Swimspeed. C). Pathlength to platform. No differences were seen between dTg mice and wt controls. Numbers on x-axis indicate training day. N = 8 M dTg, 7 F dTg, 8 M wt, 7 F wt controls. Mean  $\pm$  SEM

Data from the visual cue phase of the BACTAS mice watermaze test revealed a significant effect of day and trial [ $F(2,52) = 20.3$ ,  $p < 0.001$  and  $F(3,78) = 7.55$ ,  $p < 0.001$  respectively] on latency; pathlength [ $(2,52) = 26.4$ ,  $p < 0.001$  and  $F(3,78) = 5.84$ ,  $p = 0.001$  respectively] and on swimspeed [ $F(2,52) = 3.41$ ,  $p = 0.040$  and

$F(3,78) = 8.44$ ,  $p < 0.001$  respectively]. Genetic status had no significant effect on any of these measures. Thus the mice could see and swim well enough to learn to escape the water and their learning improved over the 3 days of visual cue training. This learning to escape to a visual cue was unaffected by the presence the of the APP & BACE1 transgenes.

During the 5 days of spatial cue training no significant effect of day  $F(4,104) = 1.45$ ,  $p = 0.22$  but an effect of trial  $F(3,78) = 4.88$ ,  $p = 0.004$  was seen for latency. The pathlength and swimspeed data analyses revealed a significant effect of day [ $F(4,104) = 11.5$ ,  $p < 0.001$  and  $F(4,104) = 6.09$ ,  $p < 0.001$  respectively] and trial [ $F(3,78) = 3.39$ ,  $p = 0.02$  and  $F(3,78) = 3.06$ ,  $p = 0.03$  respectively]. Genetic status had no significant effect on any of these measures [latency  $F(1,26) = 0.89$ ,  $p = 0.35$  (Figure 3.10A); pathlength  $F(1,26) = 0.08$  (Figure 3.10B),  $p = 0.78$ ; swimspeed  $F(1,26) = 0.35$ ,  $p = 0.56$  (Figure 3.10C)]. The reduction in escape pathlength over the 5 days of visual cue training indicates that the mice learned to escape the maze. The presence of APP and BACE1 transgenes had no impact on this learning.



**Figure 3.11 BACTAS dTg mouse memory retention in a probe trial**

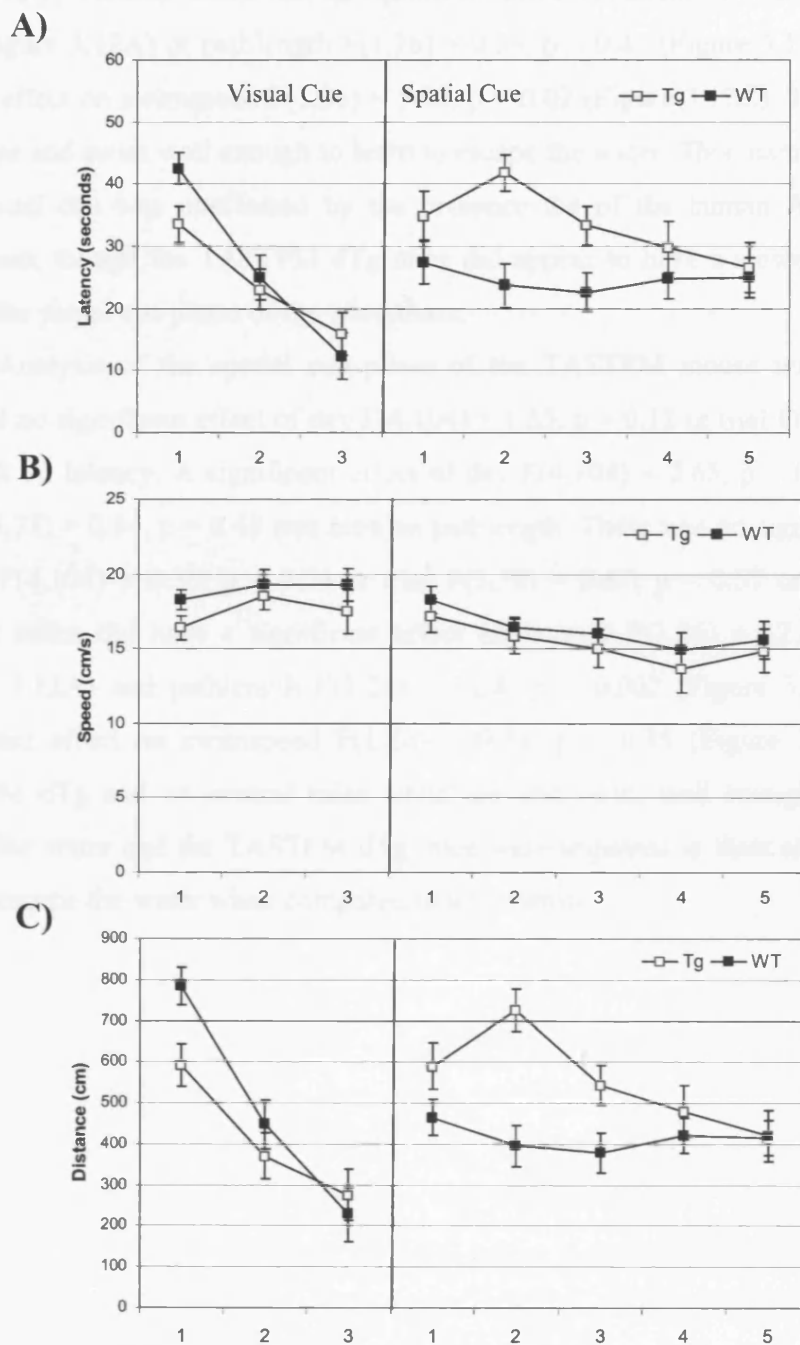
A). Percent time in quadrant B). Number of platform crossings.. No differences were seen between dTg mice and wt controls. N = 8 M dTg, 7 F dTg, 8 M wt, 7 F wt controls. Mean  $\pm$  SEM.



When tested for memory retention in a probe trial both the BACTAS and wt control mice showed a significant preference for training target quadrant [ $F(1,26) = 8.98$ ,  $p = 0.006$  and  $F(1,26) = 14.7$ ,  $p = 0.001$  respectively] showing the mice had remembered the position of the platform that they had been trained to find (Figure 3.11A). The memory retention of the mice was also measured by the number of times the mouse swam over the position in which the platform used to be (platform crossings). This measures the ability of the mice to recall the precise position of the target platform and represents a more difficult task for the mice. The BACTAS mice and wt controls showed a significant preference for the training target platform [ $F(1,26) = 10.9$ ,  $p = 0.003$  and  $F(1,26) = 10.7$ ,  $p < 0.001$  respectively] showing they had retained an accurate memory of position of the platform that they had been trained to find (Figure 3.11B). Thus, while the mice remembered how to solve the task, addition of human APP and BACE1 transgenes had no effect on this memory retention.

As the BACTAS mice did not differ from wt controls in their learning and memory they were tested for differences in synaptic plasticity. After testing the memory retrieval of these mice they were retrained to the escape the maze from the original platform position in a single training trial. There was a significant effect of trial on the latency to platform  $F(3,78) = 7.96$ ,  $p < 0.001$ , swimspeed  $F(3,78) = 13.3$ ,  $p < 0.001$  and pathlength to platform  $F(3,78) = 6.25$ ,  $p = 0.001$  during this retraining and showing the mice successfully learned to escape the maze again. Genotype had no effect on latency  $F(1,26) = 0.19$ ,  $p = 0.67$ , swimspeed  $F(1,26) = 0.53$ ,  $p = 0.47$  or pathlength  $F(1,26) = 0.08$ ,  $p = 0.79$  during retraining (Figure 3.10A,B,C). Subsequently the mice were tested for their ability to forget this learned platform position and re-learn a new platform position as a measure of synaptic plasticity. In this test the platform position was reversed to the opposite side of the pool. Analysis of the reversal training data revealed a significant effect of trial on the latency to platform  $F(4,104) = 9.83$ ,  $p < 0.001$ , swimspeed  $F(4,104) = 10.3$ ,  $p < 0.001$  and pathlength to platform  $F(4,104) = 7.93$ ,  $p < 0.001$  showing the mice successfully learned to escape the maze from the new platform position. Genotype had no effect on latency  $F(1,26) = 2.15$ ,  $p = 0.154$ , swimspeed  $F(1,26) = 1.09$ ,  $p = 0.31$  or pathlength  $F(1,26) = 0.07$   $p = 0.83$  during reversal testing (Figure 3.10A,B,C). Thus, having learned and remembered the platform position both the BACTAS dTg mice

and wt controls showed they could re-learn the platform position and then learn to escape the water from a completely new platform position.



**Figure 3.12 Learning in TASTPM mice during watermaze training**

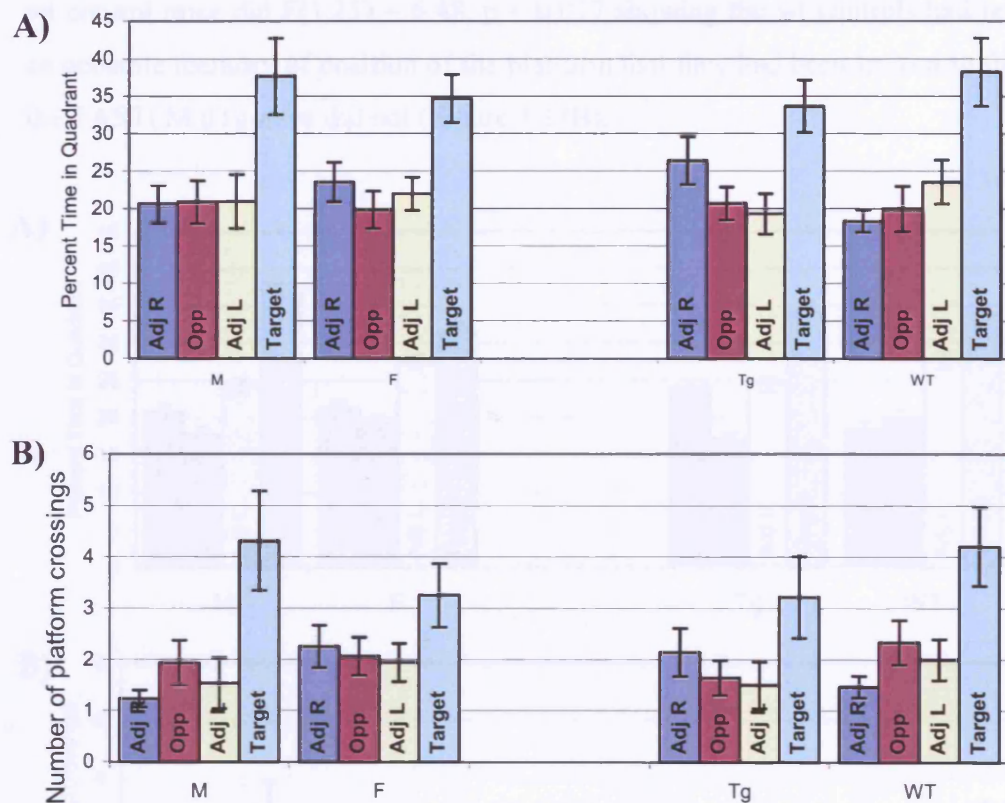
**A).** Latency to platform, **B).** Swimspeed. **C).** Pathlength to platform. No differences were seen between dTg mice and wt controls. Numbers on x-axis indicate training day. N = 7 M dTg, 8 F dTg, 7 M wt, 8 F wt controls. Mean  $\pm$  SEM

Data from the visual cue phase of the TASTPM mouse watermaze test revealed a significant effect of day  $F(2,52) = 51.2$ ,  $p < 0.001$  and trial  $F(3,78) = 12.5$ ,



$p < 0.001$  on latency and on pathlength [ $F(2,52) = 50.7$ ,  $p < 0.001$  and  $F(3,78) = 12.4$  respectively], but not on swimspeed [ $F(2,52) = 51.2$ ,  $p < 0.001$  and  $F(3,78) = 12.5$  respectively]. Genetic status had no significant effect on latency  $F(1,26) = 0.01$ ,  $p = 0.92$  (Figure 3.12A) or pathlength  $F(1,26) = 0.53$ ,  $p = 0.47$  (Figure 3.12B) but there was an effect on swimspeed  $F(1,26) = 5.97$ ,  $p = 0.02$  (Figure 3.12C). Thus the mice could see and swim well enough to learn to escape the water. This learning to escape to a visual cue was unaffected by the presence the of the human APP and PS1 transgenes, though the TASTPM dTg mice did appear to have a slower swimspeed during the visual cue phase of the watermaze.

Analysis of the spatial cue phase of the TASTPM mouse watermaze test revealed no significant effect of day  $F(4,104) = 1.85$ ,  $p = 0.12$  or trial  $F(3,78) = 1.04$ ,  $p = 0.38$  on latency. A significant effect of day  $F(4,104) = 2.65$ ,  $p = 0.037$  but not trial  $F(3,78) = 0.84$ ,  $p = 0.48$  was seen on pathlength. There was no significant effect of day  $F(4,104) = 0.36$ ,  $p = 0.84$  or trial  $F(3,78) = 0.67$ ,  $p = 0.57$  on swimspeed. Genetic status did have a significant effect on latency  $F(1,26) = 12.9$ ,  $p = 0.001$  (Figure 3.12A) and pathlength  $F(1,26) = 11.8$ ,  $p = 0.002$  (Figure 3.12B) but no significant effect on swimspeed  $F(1,26) = 0.89$ ,  $p = 0.35$  (Figure 3.12C). Thus TASTPM dTg and wt control mice could see and swim well enough to learn to escape the water and the TASTPM dTg mice were impaired in their ability to learn how to escape the water when compared to wt controls.



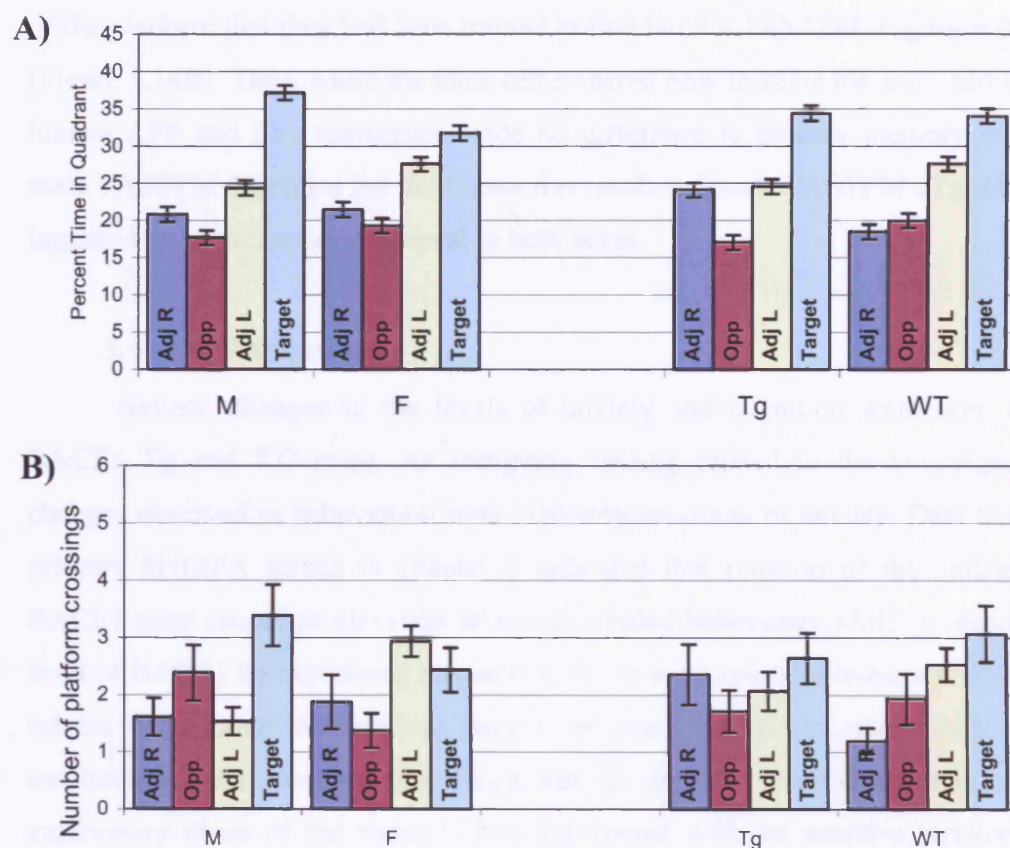
**Figure 3.13 TASTPM dTg mouse memory retention during first probe trial**

**A).** Percent time in quadrant **B).** Number of platform crossings. No differences were seen between dTg mice and wt controls. N = 8 M dTg, 7 F dTg, 8 M wt, 7 F wt controls. Mean  $\pm$  SEM.

In a test of memory retention, analysis of the percent time in the target quadrant revealed a significant preference for training target quadrant by the TASTPM  $F(1,25) = 4.25$ ,  $p = 0.05$  and wt control mice  $F(1,25) = 10.2$ ,  $p = 0.004$  (Figure 3.13A) showing the mice had remembered the target quadrant of the platform that they had been trained to find. Thus, while the mice remembered how to solve the task, addition of human APP and PS1 transgenes had no effect on this memory. Interestingly a 2 factor ANOVA of the percent time spent in target quadrant revealed a significant gender  $\times$  genotype interaction  $F(1,25) = 4.51$ ,  $p = 0.04$ . A planned comparison of this data revealed that female TASTPM dTg mice did not show a preference for the target quadrant  $F(1,25) = 1.44$ ,  $p = 0.42$  but female wt littermate control mice did  $F(1,25) = 4.87$ ,  $p = 0.037$ .

The memory retention of the mice was also measured by the number of platform crossings made during the probe trial. The TASTPM mice did not show a significant preference for the training target platform  $F(1,25) = 2.67$ ,  $p = 0.12$  and the

wt control mice did  $F(1,25) = 6.48$ ,  $p = 0.017$  showing the wt controls had retained an accurate memory of position of the platform that they had been trained to find but the TASTPM dTg mice did not (Figure 3.13B).



**Figure 3.14 TASTPM dTg mouse memory retention during second probe trial**  
A). Percent time in quadrant B). Number of platform crossings. dTg mice were impaired in their memory of the platform position.  $N = 8$  M dTg, 7 F dTg, 8 M wt, 7 F wt controls. Mean  $\pm$  SEM

In the second probe trial test 14 days after the training, the TASTPM dTg and wt control mice showed a significant preference for training target quadrant [ $F(1,25) = 12.2$ ,  $p = 0.002$  and  $F(1,25) = 10.9$ ,  $p = 0.003$  respectively, Figure 3.14A] showing the TASTPM dTg and wt control mice had remembered the target quadrant of the platform that they had been trained to find. As in the first probe trial a planned comparison of the percent time in target quadrant data from female mice revealed that female TASTPM dTg mice did not show a preference for the target quadrant  $F(,25) = 2.32$ ,  $p = 0.14$  but female wt littermate control mice did  $F(,25) = 4.31$ ,  $p = 0.048$ .

When the number of platform crossings during the probe trial was analysed the TASTPM dTg mice did not show a significant preference for training target platform  $F(1,25) = 1.36$ ,  $p = 0.25$  and the wt control mice did  $F(1,25) = 4.49$ ,  $p = 0.04$  showing the wt control mice had retained an accurate memory of the position of the platform that they had been trained to find but the TASTPM dTg mice did not (Figure 3.14B). Thus, while the mice remembered how to solve the task, addition of human APP and PS1 transgenes made no difference to broader memory recall in male TASTPM dTg mice but did impair this recall in female TASTPM dTg mice and impaired accurate memory retrieval in both sexes.

### **3.4 Discussion**

Several changes in the levels of anxiety and cognition were seen in the BACE1 Tg and KO mice. An intriguing finding related to the two significant changes observed in behavioural tests exploring measures of anxiety. Data from the primary SHIRPA screen in chapter 2 indicated that removal of the endogenous BACE1 gene caused an elevation of anxiety related behaviours while increasing the level of BACE1 by expressing human BACE1 in mice caused a reduction in anxiety related behaviours. When these mice were examined in the open field test of emotionality and elevated plus maze test of anxiety, tests which rely on the exploratory drive of the mouse when confronted with an aversive environment, namely the centre of an open field or the open arms of a plus maze (306), the Tg mice were found to be not significantly different from wt controls. They did spend 26% more time in the open arms of the elevated plus mice and had approximately 60% fewer incidents of 'stretch attend' behaviour, an indicator of increased anxiety. These results, in combination those from chapter 2, indicate a reduced anxiety phenotype for the Tg mice. In identical tests the BACE1 KO mice travelled almost 40% less distance and spent just under 50% less time in the inner zone of an open field compared to wt controls indicating an anxious phenotype. These mice also exhibited almost double the number of grooming bouts and showed a trend for less time on the open arms of the elevated plus maze when compared to wt controls. Stress-induced self grooming seen on the elevated plus maze has been shown to be a stable behavioural trait marker in rats subjected to acute stress (307). However chronic mild stress in rats also leads to a decrease in grooming behaviour over a

number of weeks (308). The BACE1 KO mice were subjected to an acute test of anxiety on the elevated plus maze and the increased incidence of grooming observed is indicative of increased anxiety in these mice. Taken together these results indicate an increased anxiety phenotype in the BACE1 KO mice. The broad phenotype of the two different lines of mice is an increased anxiety with reduced exploration in the KO mice and a reduced anxiety with increased exploration in the Tg mice. Locomotor deficits can confound these results but the data presented in chapter 2 showed that both lines of mice have unimpaired locomotion as measured by Rotarod and locomotor activity boxes.

The object recognition test investigates memory processing that relies on intact function of the peri-rhinal and post-rhinal cortices. While there was an overall preference for the novel object over the familiar in the object recognition tests for BACE1 Tg and KO mouse lines, genotype or gender had no effect on this preference. This test was designed to reveal deficits in memory retention of the familiar object and the result indicated no impairment in peri-rhinal or post-rhinal cortical memory function for BACE1 Tg or KO mice.

The activity of the mice in the fear conditioning test chamber was used to as a correlate of the freezing response, a measure of memory recall of a conditioned stimulus in the mice. An investigation of the behaviour of these mice in the fear conditioning test revealed that the BACE1 Tg mice were less active during habituation to the test chambers. Specifically the male BACE1 Tg mice had a reduced activity but the female mice did not. This difference in activity carried through to the context phase of the test. The difference in habituation activity may confound the test if the mice do not habituate uniformly to the test chamber as any subsequent differences in activity may not be attributed to a fear response alone. However no difference was found in the ratio of inactivity in the old and new contexts, the contextual fear ratio, which removes differences in baseline activity. Interestingly no differences were seen in the auditory cue phase of the test. Overall the BACE1 Tg mice had no impairment in hippocampal or amygdala function when assessed in the fear conditioning test. BACE1 KO mice had no differences in habituation to the fear conditioning chambers. They did have reduced inactivity when replaced into a test chamber with the same context in which they experienced the aversive experience of an electrical foot-shock. However they did not differ in activity levels when exposed to the test chamber with a new context. The contextual



fear ratio showed an impaired response for male but not female KO mice. When given an auditory cue that was previously associated with the old context and footshock, only male BACE1 KO mice showed impairment, females were no different from wt controls. This result reflects that seen in the contextual fear ratio with male but not female mice having an impaired cued memory recall. Hence male but not female BACE1 KO mice demonstrated impaired hippocampal and amygdala function in the fear conditioning test. It is interesting that the male BACE1 KO mice were found to be more anxious than wt controls and the females were not. No differences in memory were seen in the relatively anxiolytic object recognition test for this line of mice. It was only the relatively anxiogenic fear conditioning test that revealed a difference in memory recall in the male BACE1 KO mice. It may be possible that the male BACE1 KO mice showed impairment in the fear conditioning test because they are more anxious and more readily froze in response to the memory of an electric footshock. Further work in alternative tests of cognition will prove useful to explain difference between male BACE1 KO mice and their male wt controls.

In order to eliminate the possibility that altering BACE1 expression caused a change in sensitivity to the electrical foot shock used in this test the mice were tested for nociception in a hotplate test. No differences were seen in this test of nociception for the BACE1 Tg or KO mice when compared to wt controls. In addition the mice did not differ in their response to the pain experienced when the middle toe of a hind paw was pinched during the primary SHIRPA detailed in chapter 2.

When compared with wild type controls in a test for spatial reference memory, the BACTAS double Tg mice were equally able to navigate to a visual cue in the watermaze when the distal room cues were occluded confirming their ability to see and swim in the maze. When tested with the room cues revealed a difference in swimspeed was seen between the BACTAS Tg and wild type mice and so the pathlength measure was used as a measure of learning. The mice learned to escape the maze with a significant daily reduction in pathlength to find the platform. The presence of both the BACE1 and APP<sub>KM670/671NL</sub> transgenes had no effect on the vision or swimming ability of the mice during visual cue training or on learning during the spatial cue training. The transgenes also had no effect on memory retention in the mice. Following training the mice had an intact memory of the target platform position and were able to re-learn a new target platform position. This

sparing of synaptic function in the BACTAS mice was not seen in the TASTPM mice. These TASTPM mice, carrying APP<sub>KM670/671NL</sub> and PS1<sub>M146L</sub> transgenes and of a similar age to the BACTAS mice, were also able to navigate to a visual cue showing vision and swimming ability were unimpaired. However spatial learning was impaired as was the accurate retention of this memory in TASTPM dTg mice when compared to wt controls. In the BACTAS mice, human BACE1 is overexpressed with mutant human APP and in the TASTPM mice, mutant human PS1 is overexpressed in addition to mutant human APP. Studies have suggested that BACE1 expression is increased in AD patients (309) and the rate limiting step for  $\beta$ -amyloid production is hypothesised to be BACE1 cleavage of APP. One consequence of increased  $\beta$ -amyloid load is decreased cognition in AD patients however the impaired cognition seen in the TASTPM mice was not reflected in the BACTAS mice suggesting that the decrease in cognitive performance is accelerated by increased mutant PS1 expression rather than increased BACE1 expression.

The lack of cognitive impairment seen when human BACE1 expression is increased on its own, or in conjunction with human APP<sub>KM670/671NL</sub>, in mouse models suggests that BACE1 is less important in the progress of cognitive decline than PS1. Removing endogenous BACE1 did lead to impaired hippocampal and amygdala related memory retention. The amygdala is involved in emotional memory as well as anxiety, thus the anxiety phenotype observed when endogenous BACE1 is removed may lead to the decrease in memory retention seen in the fear conditioning test. Clearly BACE1 has other functions distinct from its activity as  $\beta$ -secretase. These data demonstrating impaired cognitive function in BACE1 KO mice is in agreement with a recent publication showing impaired cognition in another BACE1 KO mouse line (263). Further work is required to define the nature of these deficits and how they may be reflected in the human population.

*Chapter 4 - The additional  
deletion of BACE2 does not  
change the BACE1 knockout  
phenotype*

## 4.1 Introduction

At approximately the same time that BACE1 was identified, the cloning of BACE2 was also reported (92;99;120-122). BACE2 is located on chromosome 21 as is the Amyloid Precursor Protein and hence it is overexpressed in Trisomy 21 (Down's Syndrome) where affected individuals develop amyloid plaques by the age of 50. BACE2 is expressed in brain but in relatively low levels compared to BACE1. While BACE1 has been shown to be  $\beta$ -secretase and mice lacking BACE1 are essentially normal, though with sex specific anxiety (310) and mildly impaired cognition, pharmacological inhibition of BACE1 activity alone as a treatment for AD may be ineffective if BACE2 activity could substitute for the lack of BACE1. Under these circumstances BACE inhibitors would need to inhibit both BACE1 and BACE2. Inhibition of  $\beta$ -Secretase is an attractive target for a disease modifying therapy in AD. Recent data from cultured cells indicate an antagonistic effect of BACE1 and BACE2 in cells that co-express these enzymes (311). If this antagonistic effect occurs *in vivo* pharmacological inhibition of BACE1 *in vivo* would require a compound highly selective against BACE2 in order to not to reduce its ability to inhibit  $\beta$ -amyloid production.

As pharmacological inhibition of BACE1 is one potential therapy for AD patients, the cognitive function of mice lacking this enzyme and the closely related BACE2 enzyme was assessed. In order to investigate the effect of removing both BACE1 and BACE2 enzymes on behaviour, a breeding regime was devised to generate a BACE1/2 double knockout (dKO) mouse line. The dKO and wt littermate control mice were tested for anxiety, home cage behaviour, motor function and cognition. Subsequently the mice were sacrificed and gross anatomy was examined.

Lines of mice lacking BACE1 or BACE2 endogenous gene expression were created on a [C57/Bl6x129Ola/Sv] hybrid background and then interbred over 2 generations of breeding to produce homozygous BACE1/2 dKO mice. These dKO mice were analysed for behavioural changes when compared to wild type (wt) controls. Male but not female dKO mice showed elevated anxiety and female but not male dKO mice showed improved locomotor function and home cage activity when compared to same sex wild type controls. Their learning was no different from wild type controls however subtle differences were found in memory retrieval in a standard reference memory test. The subtle memory deficit and gender specific

changes in anxiety and locomotion aside, very few differences could be detected between the dKO mice and their unmodified controls.

## ***4.2 Materials and Method***

### **4.2.1 Animals**

The BACE1 KO mice were generated as previously reported (310). Briefly Exon 1 of the BACE1 genomic locus was replaced with a LacZ reporter gene (appendix 7.1.2). The BACE2 KO mice were generated by using similar cloning technology with a LacZ reporter gene inserted into the BACE2 genomic locus (appendix 7.1.3).

The BACE1/2 dKO mice for experimentation were generated with a specific breeding regime. The BACE1 KO mice and BACE2 KO mice lines were generated on a [C57Bl/6x129Ola/Sv] hybrid background and independently bred to homozygosity. The single homozygous KO mouse lines were then interbred to produce double heterozygous KO mice. These mice were subsequently interbred to produce a range of genotypes in sufficient numbers for experimental investigation. This breeding programme had the great advantage of generating the desired BACE1/2 double homozygous KO mice as well as littermates that were not carrying any form of the gene targeted alleles. The background strain was a hybrid mix of C57Bl/6 and 129Ola/Sv mouse strains.

### **4.2.2 Behavioural analysis**

#### **4.2.2.1 Anxiety**

The dKO and wt control mice were tested for anxiety using the elevated plus maze test of anxiety as described in chapter 3. This test relies on the innate behaviour of the mouse. The natural inquisitive nature of the mouse is balanced by a natural fear of open, exposed spaces. The time spent on the open and closed arms of the maze is recorded as a measure of anxiety.

#### **4.2.2.2 Home Cage Behaviours**

A range of behaviours was recorded using the Laboratory Animal Behaviour Observation, Registration and Analysis System - LABORAS<sup>TM</sup> (312), (Metris,



Hoofdorpp, Netherlands) to establish any effect of removing BACE1 and BACE2 enzymes on home cage activity. Animals were placed in the test apparatus where the vibrations created by their movements were monitored through vibration sensitive sensors. These vibrations were converted into electrical signals and processed into specific behaviours using validated algorithms. The mice were monitored for 16 hours during which the mice had access to food and water *ad libitum*. The duration and frequency of climbing, locomotion, drinking, eating, immobility and grooming were recorded.

#### **4.2.2.3 Motor Function**

Locomotor co-ordination of the mice was measured using a Rotarod (313). This consisted of a rotating drum, 15 cm in diameter that accelerated from 4 to 40 rpm over 5 minutes. The mice were placed on top of the drum, separated from each other by a divider and the drum started rotating. Each mouse was given 3 trials and the mean of these three trials was used in subsequent calculations.

#### **4.2.2.4 Cognition**

The mice were assessed for cognition in a test of spatial learning and memory based on the Morris Water Maze and adapted for mice. The maze layout was identical to that used to test the mice described in chapter 3. Dimensions and cues were identical as was the tracking system. The protocol was also identical for the visual and spatial cued training phases. The memory retention consisted of one 60 second probe trial followed by 4 rewarded spatial cue training trials to the original platform position. The following day the mice were given another single training trial to the original platform position followed by 4 more trials with the platform in the opposite quadrant (reversal), thus testing their ability to re-learn the reversed position of the platform.

### **4.2.3 Pathology**

In order to assess the effect of removing BACE1 and BACE2 proteins on gross anatomy mice were euthanased after behaviour testing and underwent a thorough post mortem examination. Organs targeted in this examination included

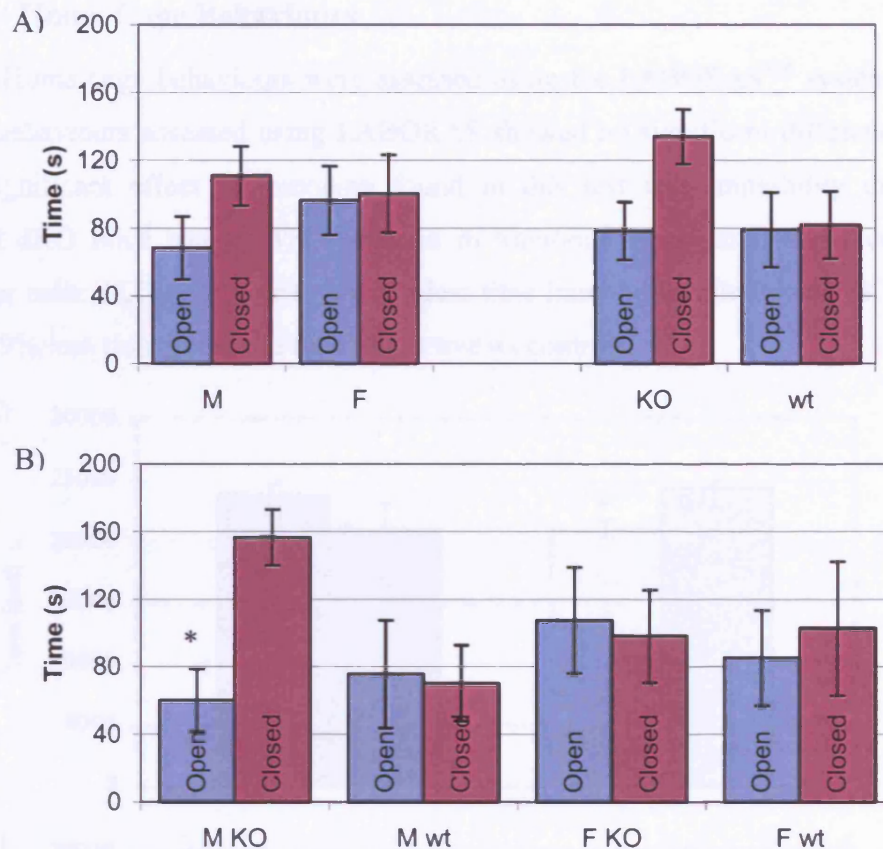
lungs, heart, liver, kidneys, spleen, stomach, duodenum, jejunum, ileum, caecum, colon, mesenteric lymph nodes, adrenal glands, thymus, testes, epididymides, prostate, seminal vesicles, ovaries, uterus, bladder, skin, tongue, larynx, oesophagus, thyroid glands, sternum, aorta, salivary gland, cervical lymph nodes, pancreas, harderian gland, eyes, spinal cord, femur, nerve, urethra, pituitary gland, gastrocnemius & triceps muscle.

### **4.3 Results**

Mice were generated with endogenous BACE1 and BACE2 genes removed on a [C57Bl/6x129Ola/Sv] hybrid background strain. The mice appeared to be healthy and normal with no problems noted during breeding. The deletion of both BACE1 and BACE2 proteins had no significant effect on the majority of behaviours when compared to wild type controls. There were subtle, gender specific effects of genotype on anxiety, home cage behaviour and locomotor function. No effect of genotype was seen on learning or re-learning ability but an effect was seen on memory retention with dKO mice showing impairment in accurate recall of the escape platform position in the watermaze.

#### **4.3.1 Anxiety**

Overall dKO mice were not significantly different from wt controls when tested in the elevated plus maze of anxiety but a clear trend for increased anxiety in the dKO mice was seen. Further analysis revealed that male dKO mice had a significant preference for the relatively safe closed arms rather than the exposed open arms of the maze indicating elevated anxiety when compared to wt controls.



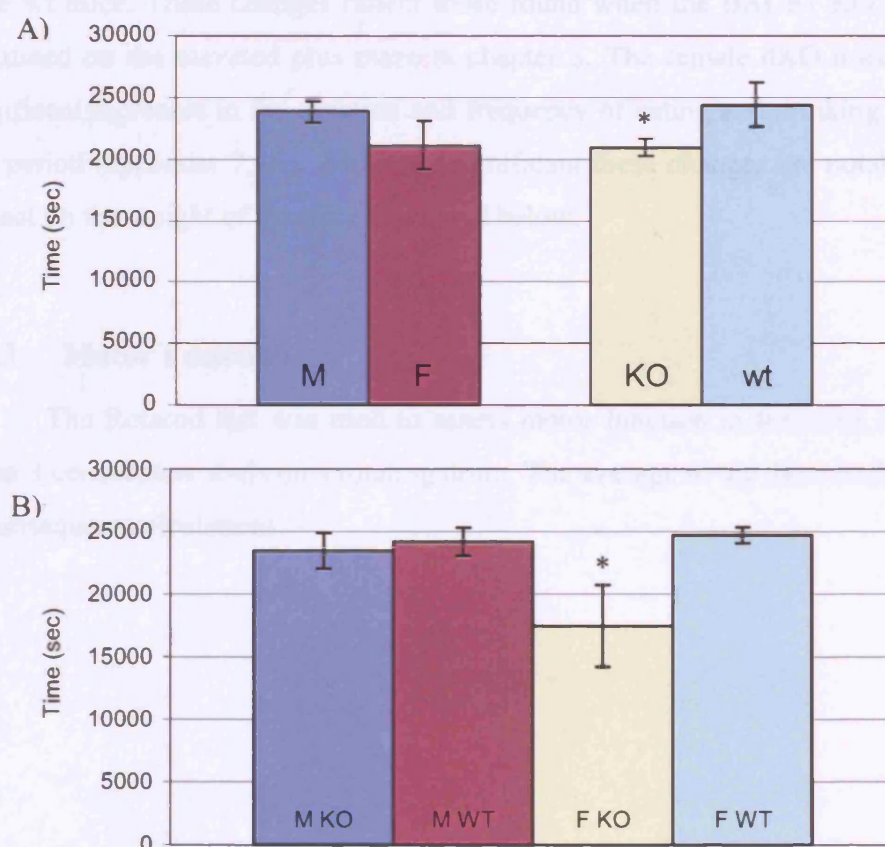
**Figure 4.1 BACE1/2 dKO mice behaviour in the elevated plus maze**

**A)** Time spent on the arms of the maze by gender or genotype. **B)** Time spent on the arms of the maze by group. Male dKO mice spent more time on the closed arms than open arms. This difference was not seen in any other group. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean  $\pm$  SEM. \*  $p < 0.05$ .

Analysis of the time spent in the open and closed arms of the elevated plus maze revealed no overall significant preference for open or closed arms by the wt control mice  $F(1,23) = 0.03$ ,  $p = 0.85$  or by the dKO mice  $F(1,23) = 1.69$ ,  $p = 0.21$  (Figure 4.1A). However when the data from individual groups of mice were analysed a significant preference for the closed arms was revealed  $F(1,23) = 5.41$ ,  $p = 0.029$  for the male dKO mice (Figure 4.1B). No preference for either arm was seen for male wt  $F(1,23) = 0.02$ ,  $p = 0.89$  or female dKO  $F(1,23) = 0.03$ ,  $p = 0.86$  or female wt mice  $F(1,23) = 0.11$ ,  $p = 0.74$ . The male dKO mice spent 160% more time on the closed arms than the open arms of the maze indicating an anxious phenotype.

### 4.3.2 Home Cage Behaviours

Home cage behaviours were assessed using the LABORAS™ system. Most of the behaviours assessed using LABORAS showed no significant difference. The only significant effect of genotype found in this test was immobility duration. Overall dKO mice had a 14% reduction in immobility compared to wt controls, however male dKO mice spent only 3% less time immobile while female dKO mice spent 29% less time immobile than respective wt controls.



**Figure 4.2 BACE1/2 dKO mouse immobility in the LABORAS™ test**

**A)** Time spent in immobility by gender or genotype. **B)** Time spent in immobility by group. Female dKO mice spent less time immobile than female wt controls. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean ± SEM. \* p < 0.05.

The LABORAS™ apparatus recorded information on 6 behaviours over a 16 hour period and only one behaviour, immobility, was found to be significantly effected by genotype  $F(1,22) = 5.16$ ,  $p = 0.033$  (Figure 4.2A). No effect of gender was found  $F(1,22) = 2.33$ ,  $p = 0.14$  however there was a near significant gender x genotype interaction  $F(1,22) = 4.03$ ,  $p = 0.057$ . A Fisher LSD post hoc test revealed a significant effect of genotype on females  $F(1,22) = 7.48$ ,  $p = 0.012$  but not males



$F(1,22) = 0.045$ ,  $p = 0.83$  (Figure 4.2B). Female mice were 29% less immobile than wt controls.

Interestingly the other behaviours measured in the LABORAS™ apparatus showed an effect of removing endogenous BACE proteins. While no significant effect of gender or genotype was seen on grooming, analysis of the number of grooming bouts of the male mice alone revealed a near significant effect of genotype  $F(1,22) = 3.72$ ,  $p = 0.06$  with male dKO mice spent 32% more time grooming than male wt mice. These changes reflect those found when the BACE1 KO mice were examined on the elevated plus maze in chapter 3. The female dKO mice had non-significant increases in the duration and frequency of eating and drinking during the test period (appendix 7.16). While not significant these changes are notable as they impact on the weight of the mice discussed below.

### 4.3.3 Motor Function

The Rotarod test was used to assess motor function in the mice. Mice were given 3 consecutive trials on a rotating drum. The average of the three trials was used in subsequent calculations.

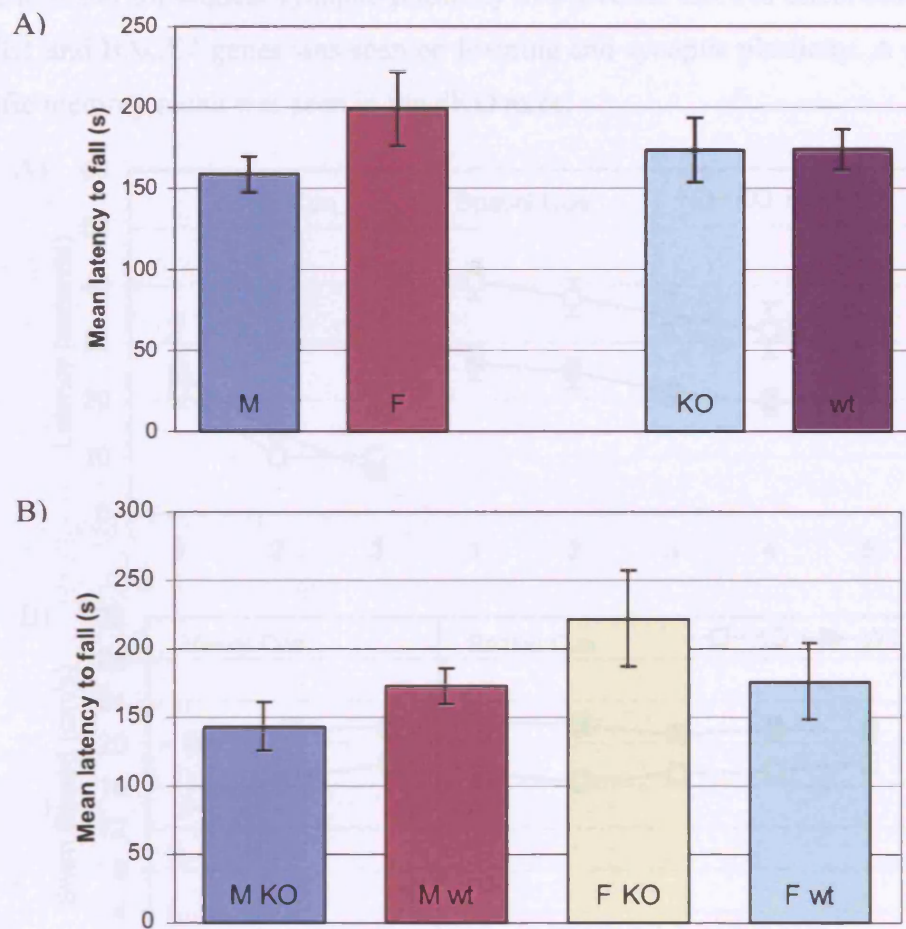
Figure 4.3.4A: The Rotarod test was used to assess motor function in the mice. Mice were given 3 consecutive trials on a rotating drum. The average of the three trials was used in subsequent calculations.

Analysis of the performance of the mice in the Rotarod test revealed no significant effect of genotype  $F(1,22) = 0.14$ ,  $p = 0.71$  and a near significant effect of gender  $F(1,22) = 3.25$ ,  $p = 0.07$  (Figure 4.3.4). The significant gender  $\times$  genotype interaction  $F(1,22) = 3.25$ ,  $p = 0.07$  was found. While no significant effect was found (Figure 4.3.4) shows that the female dKO mice were able to stay on the Rotarod an average of 20% longer than female wt controls, while the male dKO mice lasted an average of 15% less time than male wt controls.

### 4.3.4 Cognition

In a standard reference test of memory the mice were trained in spatial task in a water maze. They were then assessed for memory of the task with a





**Figure 4.3 BACE1/2 dKO mice motor co-ordination in the Rotarod test**

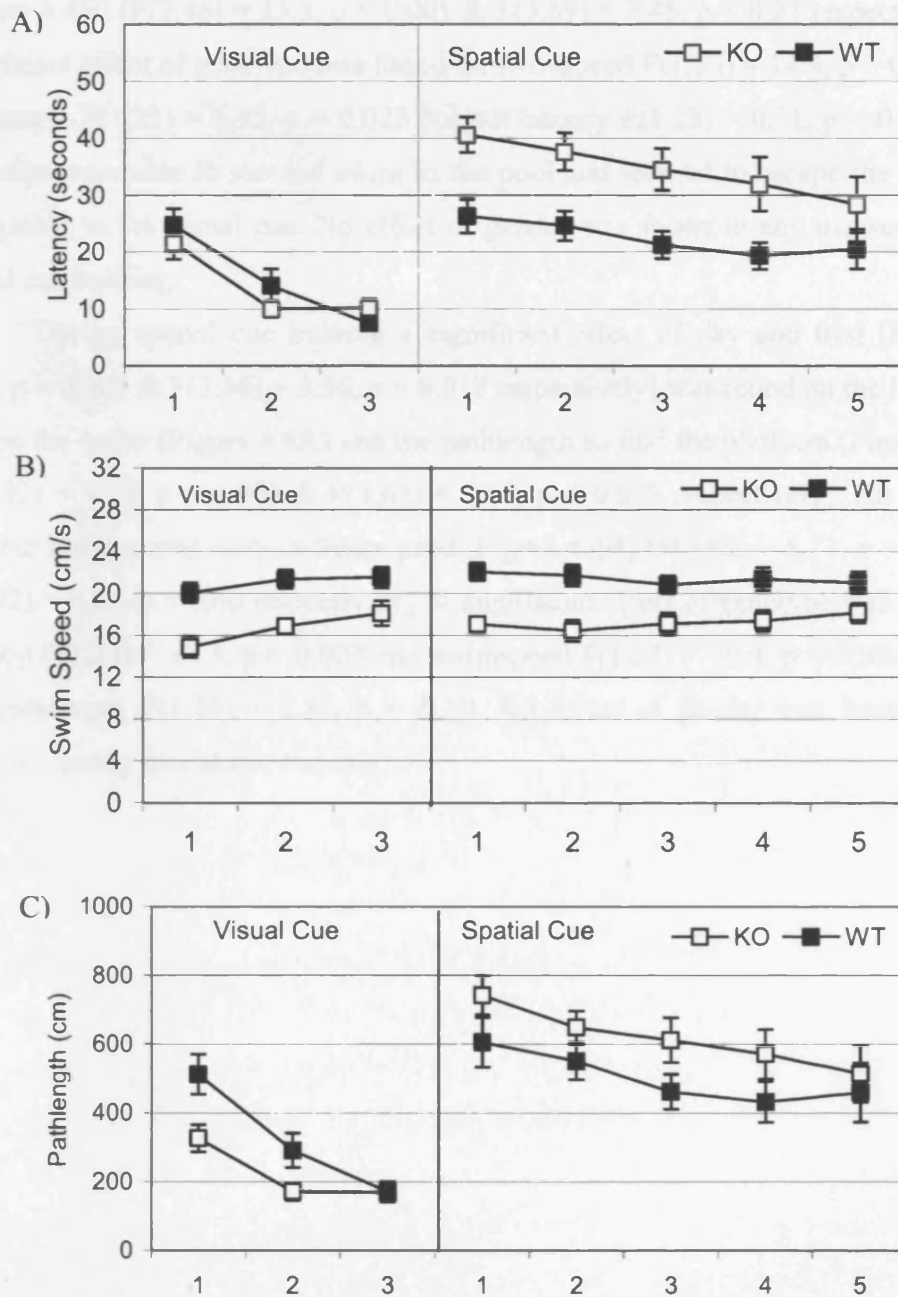
**A)** Mean latency to fall by gender or genotype. **B)** Mean latency to fall by group. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean  $\pm$  SEM.

Analysis of the performance of the mice in the Rotarod test revealed no significant effect of genotype  $F(1,23) = 0.14$ ,  $p = 0.71$  but a near significant effect of gender  $F(1,23) = 3.47$ ,  $p = 0.07$  (Figure 4.3A). No significant gender  $\times$  genotype interaction  $F(1,23) = 2.89$ ,  $p = 0.10$  was found. While no significant effect was found Figure 4.3B shows that the female dKO mice were able to stay on the Rotarod an average of 26% longer than female wt littermate control mice while male dKO mice lasted an average of 16% less time than male wt controls.

#### 4.3.4 Cognition

In a standard reference test of memory the mice were trained to spatial cues in a watermaze. They were then assessed for memory storage and retention with a

probe trial and subsequent synaptic plasticity in a reversal test. No effect of removing BACE1 and BACE2 genes was seen on learning and synaptic plasticity. A deficit in specific memory recall was seen in the dKO mice.



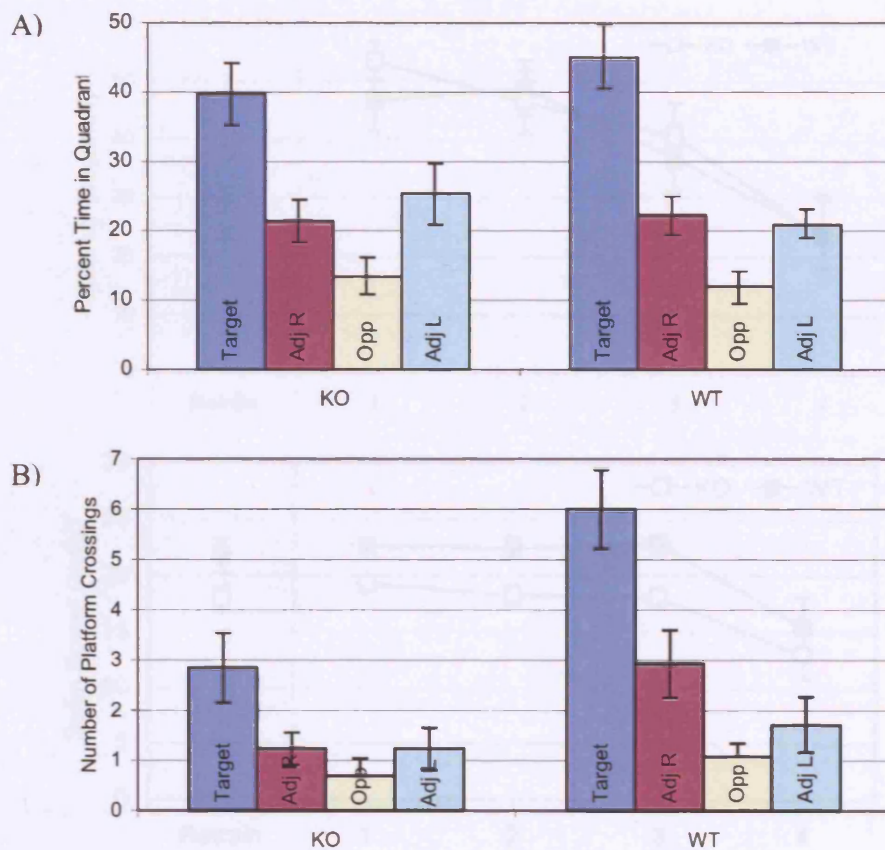
**Figure 4.4 BACE1/2 dKO mouse learning in watermaze training**

A). Latency to platform B). Swimspeed. C). Pathlength to platform. No differences were seen between dKO mice and wt controls. Numbers on x-axis indicate training day. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean  $\pm$  SEM

Analysis of performance of the BACE1/2 dKO during visual cue training revealed a significant effect of day and trial [ $F(2,46) = 29.1$ ,  $p < 0.001$  &  $F(3,69) =$

4.96,  $p = 0.004$  respectively] on the latency to escape the water (Figure 4.4A) and the pathlength to find the platform (Figure 4.4C) [ $F(2,46) = 23.8$ ,  $p < 0.001$  &  $F(3,69) = 4.39$ ,  $p = 0.007$  respectively]. An effect of day but not trial was seen on swimspeed (Figure 4.4B) [ $F(2,46) = 13.3$ ,  $p < 0.001$  &  $F(3,69) = 1.45$ ,  $p = 0.23$  respectively]. A significant effect of genotype was found on swimspeed  $F(1,23) = 14.4$ ,  $p = 0.001$  and pathlength  $F(1,23) = 5.95$ ,  $p = 0.023$  but not latency  $F(1,23) = 0.11$ ,  $p = 0.73$ . Thus the mice were able to see and swim in the pool and learned to escape the water by navigating to the visual cue. No effect of gender was found in any measure during visual cue training.

During spatial cue training a significant effect of day and trial [ $F(4,92) = 4.27$ ,  $p = 0.003$  &  $F(3,69) = 3.56$ ,  $p = 0.018$  respectively] was found on the latency to escape the water (Figure 4.4A) and the pathlength to find the platform (Figure 4.4C) [ $F(4,92) = 4.28$ ,  $p = 0.003$  &  $F(3,69) = 3.59$ ,  $p = 0.018$  respectively]. An effect of trial but not day was seen on Swim peed (Figure 4.4B) [ $F(3,69) = 5.11$ ,  $p = 0.003$  &  $F(4,92) = 0.69$ ,  $p = 0.60$  respectively]. A significant effect of genotype was found on latency  $F(1,23) = 11.3$ ,  $p = 0.003$  and swimspeed  $F(1,23) = 10.4$ ,  $p = 0.004$  and but not pathlength  $F(1,23) = 2.88$ ,  $p = 0.10$ . No effect of gender was found in any measure during spatial cue training.

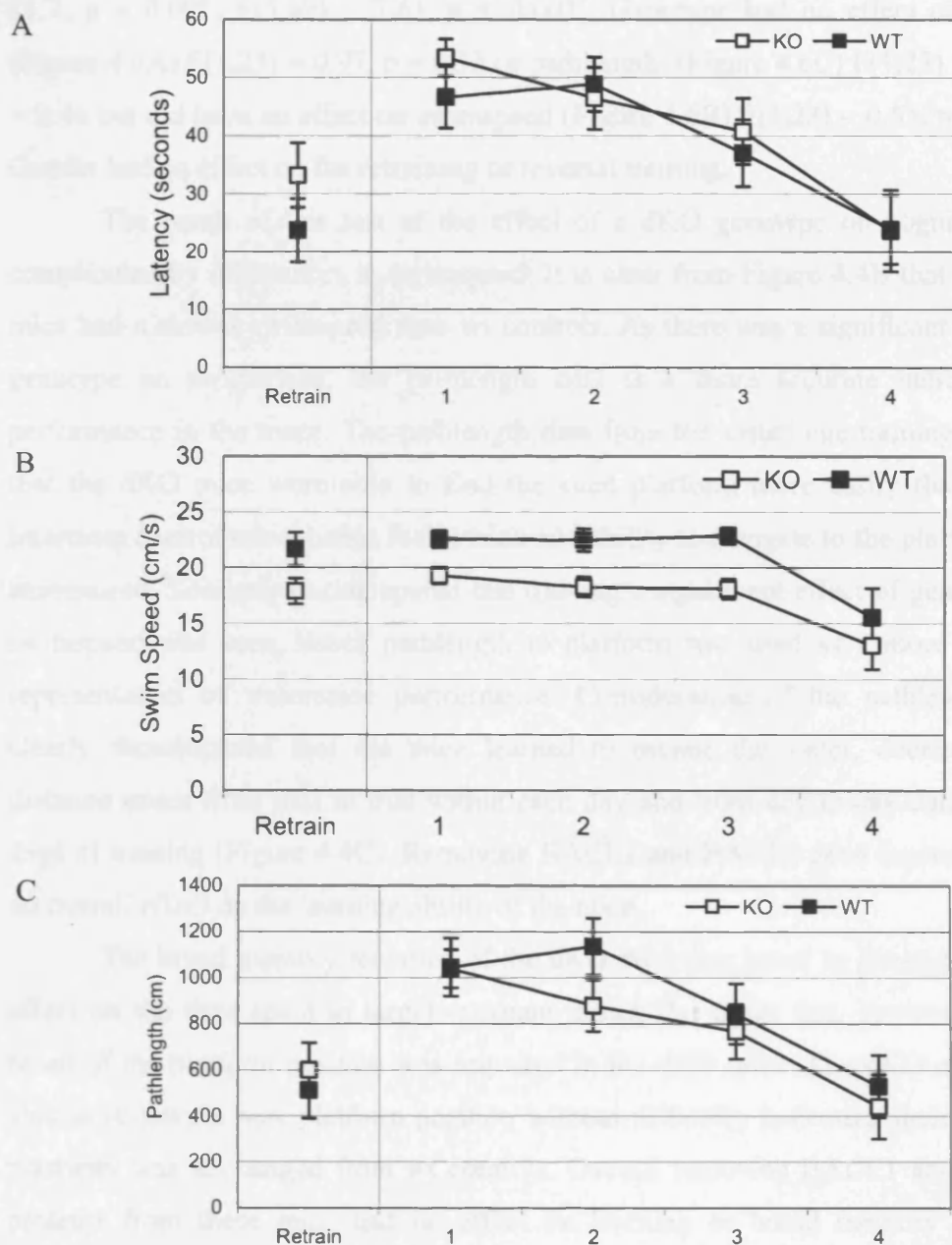


**Figure 4.5 BACE1/2 dKO mouse memory retention in a probe trial**

**A)** Percent time in platform quadrant. **B)** Number of platform crossings. dKO mice did not show a significant preference for the target platform. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean  $\pm$  SEM

The time spent in quadrant during the test of memory retention was analysed and both the dKO and wt mice showed a significant preference for the training target quadrant  $F(1,23) = 9.09$ ,  $p = 0.006$  and  $F(1,23) = 20.0$ ,  $p < 0.001$  respectively (Figure 4.5A). However analysis of the number of platform crossings, a more precise measure of memory recall, showed that while wt littermate control mice showed a significant preference for the target platform  $F(1,23) = 18.9$ ,  $p < 0.001$ , the dKO mice did not  $F(1,23) = 2.83$ ,  $p = 0.11$  (Figure 4.5B). Gender had no effect on this memory retention.





**Figure 4.6 BACE1/2 dKO mouse synaptic plasticity in a reversal test**

A). Latency to platform B). Swimspeed. C). Pathlength to platform. No differences were seen between dKO mice and wt controls. Numbers on x-axis indicate training trial. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean  $\pm$  SEM

After memory retention was tested the mice were given one retraining trial to the original platform position. No effect of genotype was found on latency, swimspeed or pathlength. Subsequently the mice were tested for memory plasticity with a session of reversal training trials. Analyses of latency, Speed and pathlength data revealed an effect of trial in each measure [ $F(3,69) = 7.83$ ,  $p < 0.001$ ;  $F(3,69) =$



11.7,  $p < 0.001$ ;  $F(3,69) = 7.63$ ,  $p < 0.001$ ]. Genotype had no effect on latency (Figure 4.6A)  $F(1,23) = 0.97$ ,  $p = 0.33$  or pathlength (Figure 4.6C)  $F(1,23) = 0.55$ ,  $p = 0.46$  but did have an effect on swimspeed (Figure 4.6B)  $F(1,23) = 0.55$ ,  $p = 0.028$ . Gender had no effect on the retraining or reversal training.

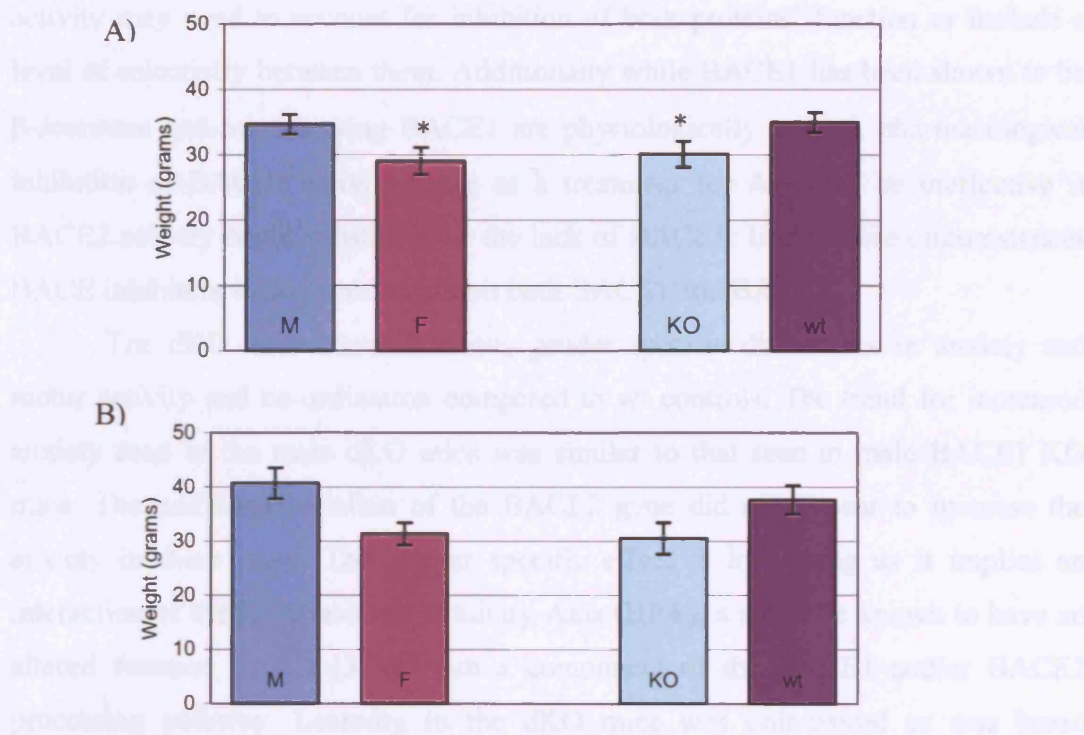
The result of this test of the effect of a dKO genotype on cognition was complicated by differences in swimspeed. It is clear from Figure 4.4B that the dKO mice had a slower swimspeed than wt controls. As there was a significant effect of genotype on swimspeed, the pathlength data is a more accurate indication of performance in the maze. The pathlength data from the visual cue training indicate that the dKO mice were able to find the cued platform more easily than the wt littermate control mice, hence their vision and ability to navigate to the platform was unimpaired. Similarly during spatial cue training a significant effect of genotype on swimspeed was seen, hence pathlength to platform was used as a more accurate representation of watermaze performance. Consideration of the pathlength data clearly demonstrated that the mice learned to escape the water, decreasing the distance swam from trial to trial within each day and from day to day during the 5 days of training (Figure 4.4C). Removing BACE1 and BACE2 gene expression had no overall effect on the learning ability of the mice.

The broad memory retention of the dKO mice was intact as genotype had no effect on the time spent in target quadrant during the probe test, however precise recall of the platform position was impaired in the dKO mice. The dKO mice were able to re-learn a new platform position without difficulty indicating their synaptic plasticity was unchanged from wt controls. Overall removing BACE1 and BACE2 proteins from these mice had no effect on learning or broad memory recall of synaptic plasticity. Effects were seen on the speed at which the mice could swim, with dKO mice swimming more slowly, and on the precise memory recall of a learned platform position.

#### **4.3.5 Pathology**

The post mortem examination was carried out at age 13 months and revealed a normal gross anatomy with no gene associated abnormalities detected. Analysis of the brain weights revealed no significant effect of gender or genotype. Interestingly

the BACE1/2 dKO mice were lighter than wt controls at 9 and 13 months of age with a greater difference in female mice than male mice.



**Figure 4.7 Weight of BACE1/2 dKO mice**

**A).** Weight at age 9 months **B).** Weight at age 13 months. At 9 months of age dKO mice were lighter than wt controls and females were lighter than males. N = 8 M dKO, 9 F dKO, 5 M wt, 5 F wt controls. Mean  $\pm$  SEM. \*  $p < 0.05$

When weighed at 9 months of age a significant effect of gender  $F(1,23) = 5.37$ ,  $p = 0.03$  and of genotype  $F(1,23) = 4.72$ ,  $p = 0.04$  was found on body weight (Figure 4.7A). At 13 months a reduced number of animals were measured and the effects of gender  $F(1,5) = 6.07$ ,  $p = 0.057$  and genotype  $F(1,5) = 4.20$ ,  $p = 0.09$  on body weight failed to reach significance (Figure 4.7B). At each age the dKO mice were lighter than wt controls (13.9% at 9 months rising to 18.9% at 13 months). As expected female mice were lighter than male mice at each age (19.3% at 9 months and 29.8% at 13 months). At 9 months of age female dKO mice were 21% lighter than female wt controls and male dKO mice were 9.6% lighter than wt controls.

#### 4.4 Discussion

This chapter presented data from an investigation of the consequences of removing endogenous BACE1 and BACE2 genes in a single line of dKO mice. Both

of these proteins cleave APP at the  $\beta$ -secretase site, an attractive target for pharmacological intervention in AD, and so any therapy based on inhibiting BACE activity may need to account for inhibition of both proteins' function or include a level of selectivity between them. Additionally while BACE1 has been shown to be  $\beta$ -secretase and mice lacking BACE1 are physiologically normal, pharmacological inhibition of BACE1 activity alone as a treatment for AD may be ineffective if BACE2 activity could substitute for the lack of BACE1. Under these circumstances BACE inhibitors would need to inhibit both BACE1 and BACE2.

The dKO mice showed subtle, gender specific differences in anxiety and motor activity and co-ordination compared to wt controls. The trend for increased anxiety seen in the male dKO mice was similar to that seen in male BACE1 KO mice. The additional deletion of the BACE2 gene did not appear to increase the anxiety in these mice. The gender specific effect is interesting as it implies an interaction of the Hypothalamic Pituitary Axis (HPA), a structure known to have an altered function in AD (314), with a component of the BACE1 and/or BACE2 processing pathway. Learning in the dKO mice was unimpaired as was broad memory recall however the memory of a specific target, namely the platform location to which the mice were trained, was poorer in dKO mice compared to wt controls suggesting impairment in the co-ordinated memory recall function of the hippocampus and neo-cortex. Interestingly the dKO mice swam more slowly than controls. This may be due to impairment in swimming ability though the motor co-ordination data suggest this is not the case. When tested in the watermaze the dKO mice were 13.9% lighter than wild type littermate control and this difference in weight may have contributed to the slower swimspeed. Both male and female dKO mice were lighter than respective wt controls. The improved motor co-ordination and decreased home cage inactivity shown by female dKO mice demonstrate that though they were lighter than controls they had a more robust motor function and were more active than wt controls. A more extensive examination of body fat and muscle composition may reveal a fuller explanation of this result. No differences in gross pathology were seen. Overall very few differences were seen in the BACE1/2 dKO mice, beyond those observed in BACE1 KO mice.

While inhibition of BACE1 in humans is an attractive target for disease modifying therapy in Alzheimer Disease, in keeping with data contained in chapters 2 and 3 of this thesis and with previously published work (310), the data might

suggest that inhibition of both BACE1 and BACE2 would lead to anxiety related behaviour in treated patients. However, one key difference is that here mice have been generated which completely lack BACE1 and BACE2 proteins while the administration of a BACE inhibitor as a therapeutic would not change protein levels, at least in the short term, only inhibit the enzyme activity. These data do suggest that in searching for a novel  $\beta$ -secretase inhibiting therapy, pharmacologists need not be overly concerned with high compound selectivity against BACE2. The anxiety phenotype seen in these mice raises the intriguing question as to an as yet undiscovered function of BACE1 and/or BACE2 in the serotonergic system and how this may relate to Alzheimer's Disease.

*Chapter 5 - BACE1 knockout  
mice fail to put on weight at  
the same rate as wild type  
control mice*



## **5.1 Introduction**

AD patients have a number of secondary behavioural indications associated with the disease including anxiety, aggression and depression. In addition weight loss has been observed in AD patients (315) and may be predictive of AD onset (316). While these symptoms can be attributed to a number of separate causes, in combination these peripheral symptoms may be explained by impaired functioning of the hormonal control by the Hypothalamic-Pituitary-Axis (HPA) (196;197). The hypothalamus and pituitary glands are located at the base of the brain in the sella turcica, a pit of the sphenoid bone. The pituitary gland secretes a number of hormones vital to various organs including growth hormone controlling, among other things, fat, bone and muscle growth. Thyroid stimulating hormone is also secreted by the pituitary gland. This has an effect on the thyroid gland and thyroxine secretion, which has a range of influences on growth. Hypothalamic regulatory hormones regulate the pituitary secretions. Both the pituitary and hypothalamic glands hormonal secretions are highly vascular and are regulated with a feedback control system via the blood stream.

BACE1 has been identified as  $\beta$ -secretase however the mature BACE1 protein has numerous binding domains (see 1.3.2) and splice variants have been localised to the pancreas (95). These features, combined with the fact that APP is described as a poor substrate for BACE1 (317), suggest additional functions for BACE1. It is highly likely therefore, that BACE1 has a function independent of APP processing. As described in the introduction to this thesis, recent publications have proposed alternative substrates for BACE1 including P-selectin glycoprotein ligand-1, the sialyl-transferase ST6Gal1, amyloid precursor like protein 2 (APLP2), lipoprotein receptor-related protein (LRP) and a voltage gated sodium channel  $\beta$ -subunit (SCN2 $\beta$ ) with the potential for other substrates yet to be discovered.

This chapter reports the findings of an investigation of the metabolic changes found in the BACE1 Tg and KO mouse lines. During the behavioural phenotyping of the mice, weight measurements were routinely taken. It was noted that a difference in weight was apparent between the BACE1 Tg and KO mouse lines and respective wild type controls. At post mortem tissues were harvested and various measurements taken to attempt to explain the differences in observed body weight.

## **5.2 *Materials and Methods***

### **5.2.1 Animals**

The animals used to measure body weights, provide material for the tissue weights and temperatures measurements were those reported in chapter 3 and were sacrificed at approximately 15 to 18 months of age. Animals used to provide tissues for the neurochemistry and blood biochemistry analyses were those described in chapter 2, aged approximately 4 to 5 months.

### **5.2.2 Body Weights**

The total body weight of the mice was measured using a standard animal balance and weighing bowl. BACE1 KO mice were weighed at averages age of 8, 13 and 15 months. The BACE1 Tg mice were weighed at average ages of 12, 16 and 18 months. Some of the mice died over the study period and half of the BACE1 Tg mice from each group were sacrificed prior to the final body weight measurements.

### **5.2.3 Tissue Weights**

At post mortem the peri-genital fat pads and the interscapular brown adipose fat pads were dissected away from surrounding tissues, cleaned of contaminating viscera and weighed. These tissues were chosen as, besides being relatively easy to identify as discrete deposits and isolate, they are indicators of fat storage and fat metabolism.

### **5.2.4 Temperature**

Temperature measurements were taken using a transponder microchip incorporating a temperature sensor that was implanted in the posterior upper left flank of each animal. The microchip was inserted and allowed to remain in place for 2 days prior to temperature readings. These measurements were taken one week prior to culling the animals.

### 5.2.5 Neurochemistry

Upon completion of the behavioural testing, brains from 4 to 5 month old BACE1 mice were harvested and the following regions were dissected and snap frozen and stored at -80°C until assay: dorsal striatum, nucleus accumbens, hippocampus, hypothalamus, cerebellum and frontal cortex (left and right hemispheres pooled). Details of the method used to examine neurochemical changes in these brains are in appendix 7.3.1. Briefly, samples were probe sonicated in buffer (10 µl/mg tissue) and centrifuged at 10,000 rpm and 4°C for 10 minutes. Supernatant fractions were analysed using HPLC with electrochemical detection and quantified by comparison with calibration curves run previously for 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT). Turnover rates were assessed by calculating the following ratios: DOPAC/DA, HVA/DA, DOPAC/HVA and 5-HIAA/5-HT. The *ex vivo* neurochemical phenotype of the animals was analysed by an analysis of variance (ANOVA). Differences between individual means were assessed with the post-hoc Fisher's test.

### 5.2.6 Blood Biochemistry

The mice were analysed for changes in blood biochemistry following the behavioural testing. Blood from the mice was collected under terminal anaesthesia. The whole blood was collected into EDTA coated tubes and spun at 13,000 rpm for 2 minutes to isolate the blood serum. This was removed and frozen at -80°C until use. The serum was analysed using published methods (318).

## 5.3 Results

### 5.3.1 Animal Ages and Survival

Throughout the period of study a number of mice died. The number of mice weighed at each time point is shown for BACE1 KO mice in Table 5.1A and BACE1 Tg in Table 5.1B. The dates of death of the mice were not recorded and so an accurate survival analysis was not possible however from a starting cohort of 24 KO and 24 wt control mice (12 of each gender), 15 KO and 21 wt control mice survived

to 15 months of age. From a starting cohort of 26 Tg and 26 wt control mice (13 of each gender), 23 Tg and 26 wt control mice survived to 16 months of age (not all of the mice were included in the final measurements for the Tg mice). These data represent a survival of 62.5% for the KO mice compared to 87.5% for the wt control mice and 88.5% for the Tg mice compared to 100% for the wt controls.

A)

Age (months)	M KO	M wt	F KO	F wt	KO	Wt	M	F
8	9	12	9	11	18	23	21	20
13	9	12	9	11	18	23	21	20
15	7	12	8	9	15	21	19	17

B)

Age (months)	M Tg	M wt	F Tg	F wt	Tg	wt	M	F
12	11	13	12	13	23	26	24	25
16	11	13	12	13	23	26	24	25
18	5	7	6	7	11	14	12	13

**Table 5.1 Number and age of BACE1 mice**

**A) KO mice. B) Tg mice**

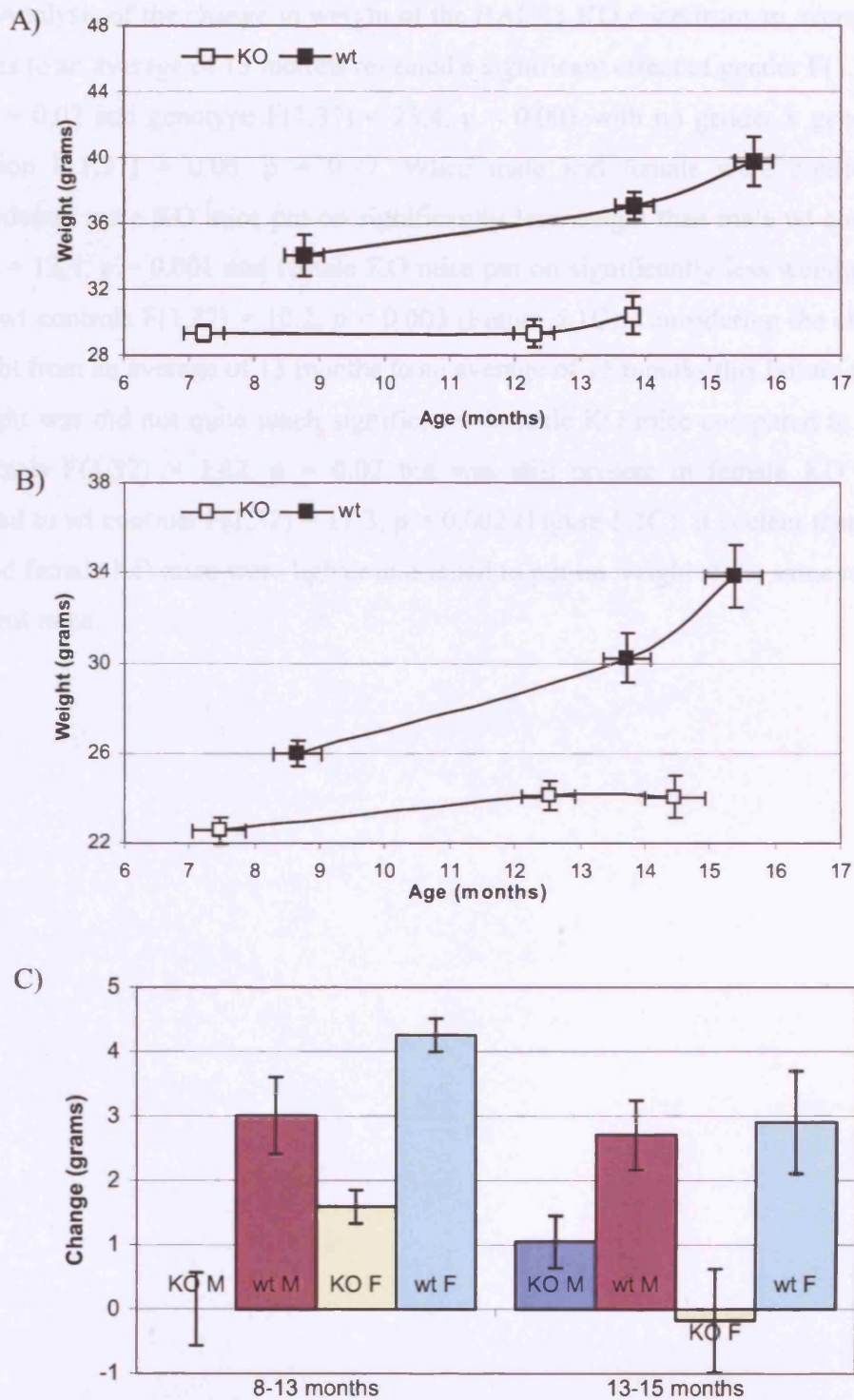
Small differences in the average ages of the mice were apparent as a consequence of the breeding regimes used to generate the experimental cohorts of mice. While the range of birth dates for the BACE1 Tg mice was 1.7 months, a 2 factor ANOVA of the age of these mice revealed no significant differences between the genders  $F(1,45) = 0.15$ ,  $p = 0.70$  or genotypes  $F(1,45) = 0.12$ ,  $p = 0.73$  investigated. No gender x genotype interaction was seen. However as a result of the independent breeding regime used for the BACE1 KO mice the dates of birth ranged over 3 months and while no significant difference between the age of the genders  $F(1,37) = 0.07$ ,  $p = 0.79$  was seen, the mean age of the different genotypes  $F(1,37) = 13.9$ ,  $p = 0.001$  did significantly differ between the KO and wt mice. No gender x genotype interaction was seen. Consequently a direct comparison between the weights of the KO and wt control mice was not feasible as the weight of the mice changed with age, however as the interval between weighing had been constant a comparison of the change in weight over time was possible. The actual weights of the BACE1 KO (Figure 5.1) and BACE1 Tg mice (Figure 5.2) are shown below in order to give a clear representation of the effect of genotype on weight. In

calculations of statistical differences the change in weight of the KO (Figure 5.1C) and Tg (Figure 5.2C) mouse lines are used.

### **5.3.2 Body Weights**

Overall the BACE1 KO mice were lighter than wt control mice and put on less weight as they aged. This failure to put on weight was more prevalent in female KO mice than male KO mice. The male but not female BACE1 Tg mice were heavier and put on more weight than wt controls.





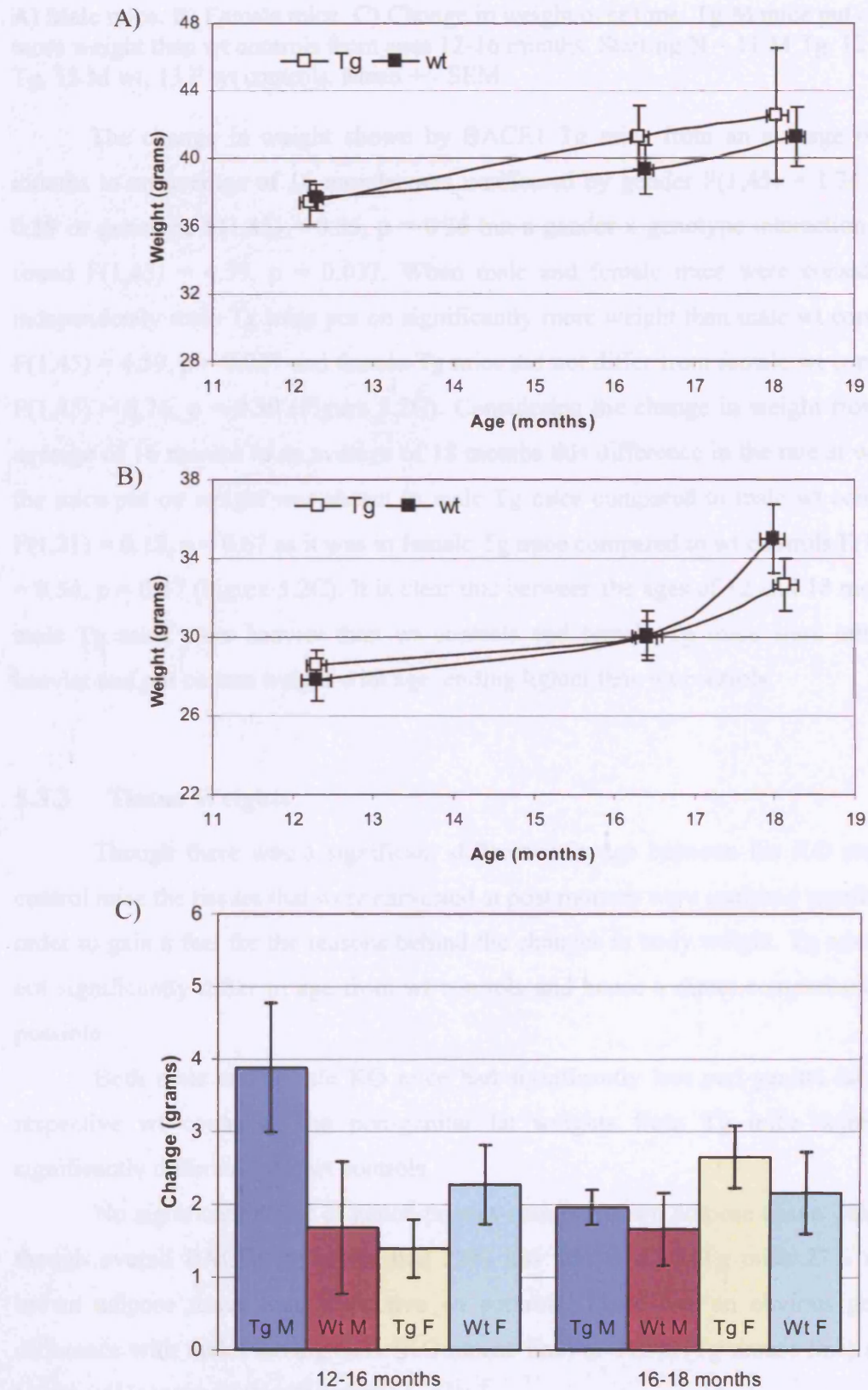
**Figure 5.1 Weights of BACE1 KO mice aged 7 – 16 months**

A) Male mice. B) Female mice. C) Change in weight over time. KO mice failed to put on weight at the same rate as wt controls. Starting N = 9 M KO, 9 F KO, 12 M wt, 12 F wt controls. Mean  $\pm$  SEM

Analysis of the change in weight of the BACE1 KO mice from an average of 8 months to an average of 13 months revealed a significant effect of gender  $F(1,37) = 5.91$ ,  $p = 0.02$  and genotype  $F(1,37) = 23.4$ ,  $p < 0.001$  with no gender x genotype interaction  $F(1,37) = 0.08$ ,  $p = 0.77$ . When male and female were considered independently male KO mice put on significantly less weight than male wt controls  $F(1,37) = 13.4$ ,  $p = 0.001$  and female KO mice put on significantly less weight than female wt controls  $F(1,37) = 10.2$ ,  $p = 0.003$  (Figure 5.1C). Considering the change in weight from an average of 13 months to an average of 15 months this failure to put on weight was did not quite reach significance in male KO mice compared to male wt controls  $F(1,32) = 3.42$ ,  $p = 0.07$  but was still present in female KO mice compared to wt controls  $F(1,32) = 11.3$ ,  $p = 0.002$  (Figure 5.1C). It is clear that both male and female KO mice were lighter and failed to put on weight at the same rate as wt control mice.



Figure 5.2 Weight of BACE1-Tg mice aged 12 – 16 months



**Figure 5.2 Weight of BACE1 Tg mice aged 12 – 18 months**



A) Male mice. B) Female mice. C) Change in weight over time. Tg M mice put on more weight than wt controls from ages 12-16 months. Starting N = 11 M Tg, 12 F Tg, 13 M wt, 13 F wt controls. Mean  $\pm$  SEM

The change in weight shown by BACE1 Tg mice from an average of 12 months to an average of 16 months was unaffected by gender  $F(1,45) = 1.74$ ,  $p = 0.19$  or genotype  $F(1,45) = 0.85$ ,  $p = 0.36$  but a gender  $\times$  genotype interaction was found  $F(1,45) = 4.59$ ,  $p = 0.037$ . When male and female mice were considered independently male Tg mice put on significantly more weight than male wt controls  $F(1,45) = 4.59$ ,  $p = 0.037$  and female Tg mice did not differ from female wt controls  $F(1,45) = 0.76$ ,  $p = 0.39$  (Figure 5.2C). Considering the change in weight from an average of 16 months to an average of 18 months this difference in the rate at which the mice put on weight was absent in male Tg mice compared to male wt controls  $F(1,21) = 0.18$ ,  $p = 0.67$  as it was in female Tg mice compared to wt controls  $F(1,21) = 0.54$ ,  $p = 0.47$  (Figure 5.2C). It is clear that between the ages of 12 and 18 months male Tg mice were heavier than wt controls and female Tg mice were initially heavier and put on less weight with age, ending lighter than wt controls.

### 5.3.3 Tissue Weights

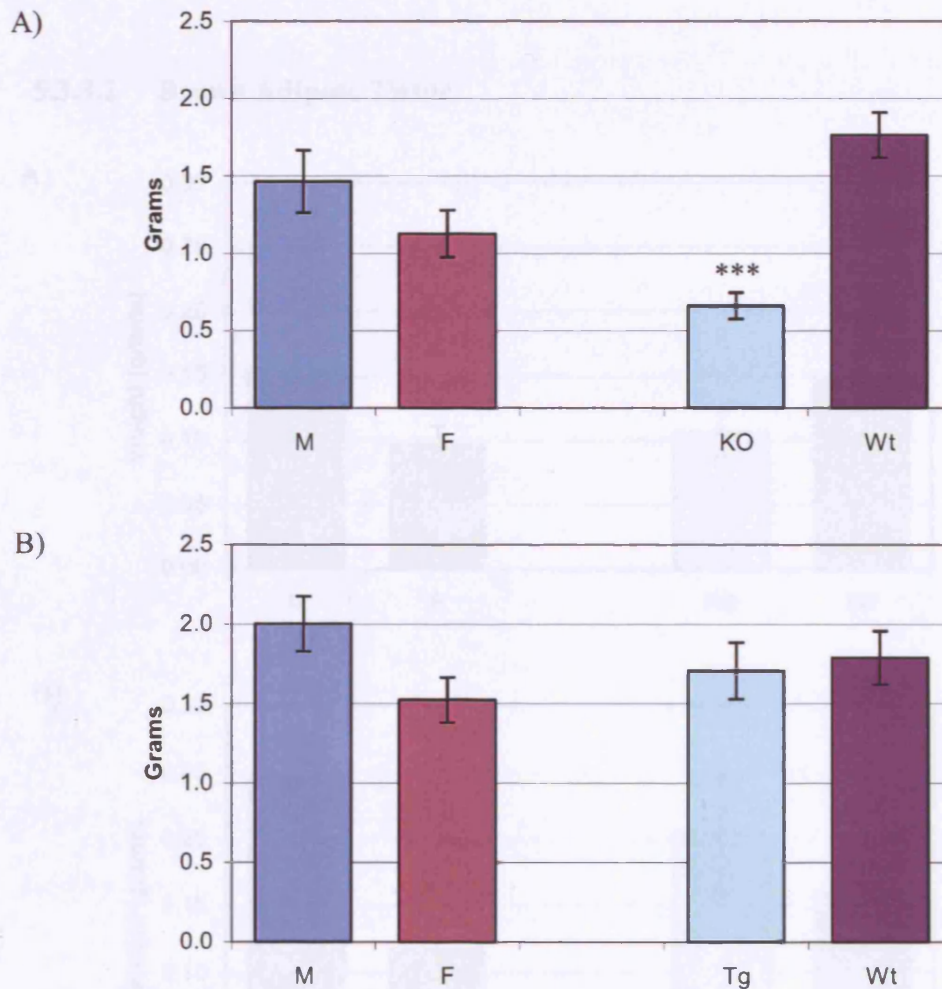
Though there was a significant difference in age between the KO and wt control mice the tissues that were harvested at post mortem were analysed together in order to gain a feel for the reasons behind the changes in body weight. Tg mice did not significantly differ in age from wt controls and hence a direct comparison was possible.

Both male and female KO mice had significantly less peri-genital fat than respective wt controls. The peri-genital fat weights from Tg mice were not significantly different from wt controls.

No significant effect of genotype was seen on brown adipose tissue weights, though overall BACE1 KO mice had 28% less and BACE1 Tg mice 27% more brown adipose tissue than respective wt controls. There was an obvious gender difference with males having 62% (KO mouse line) or 110% (Tg mouse line) more brown adipose tissue than females.

The weights of the brains from BACE1 Tg mice were no different from those of wt controls.

### 5.3.3.1 Peri-Genital Fat



**Figure 5.3 BACE1 Peri-Genital Fat weights**

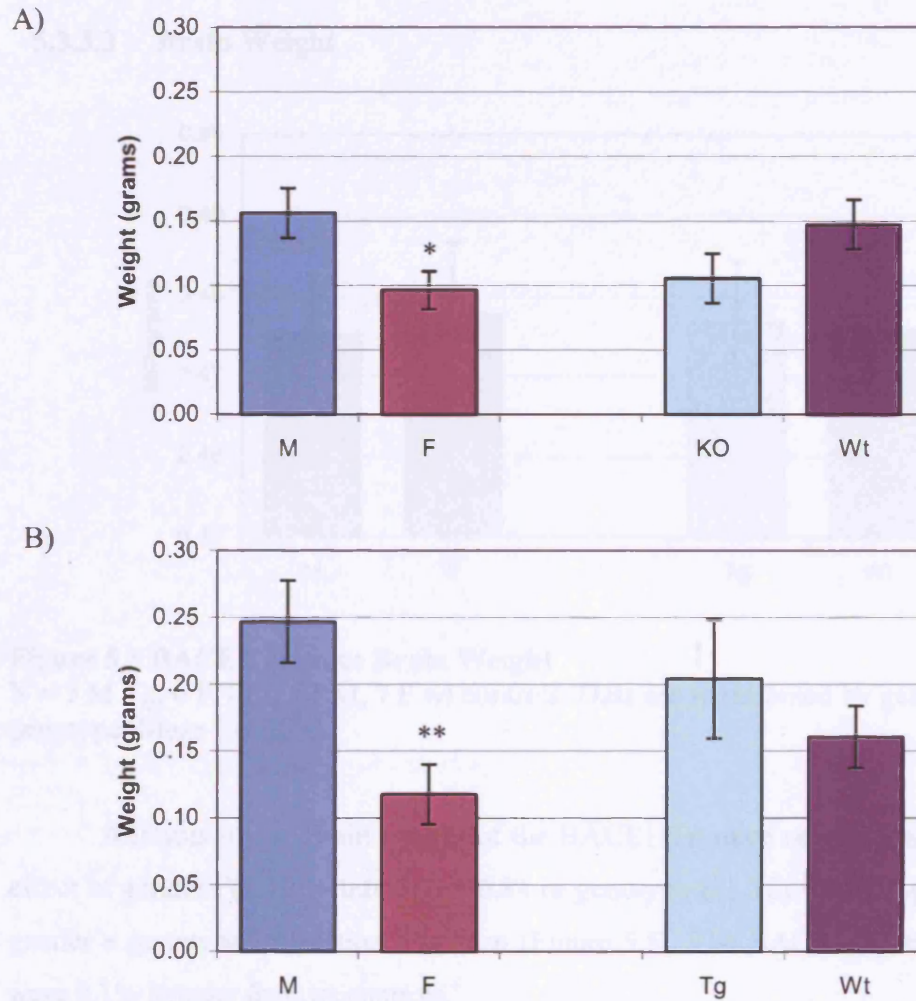
**A)** KO mice. **B)** Tg mice. N = 7 M KO, 8 F KO, 12 M wt, 9 F wt controls. N = 5 M Tg, 6 F Tg, 7 M wt, 7 F wt controls. Data are represented by gender or by genotype. Mean +/- SEM. \*\*\* p < 0.001

Analysis of the Peri-Genital Fat (PGF) weight of BACE1 KO mice revealed no significant effect of gender  $F(1,32) = 1.17$ ,  $p = 0.29$  but an effect of genotype  $F(1,32) = 32.1$ ,  $p < 0.001$  (Figure 5.3A). In a planned comparison of PGF weight, male and female KO mice had significantly lighter PGF weight than respective wt controls [ $F(1,32) = 20.8$ ,  $p < 0.001$  and  $F(1,32) = 11.9$ ,  $p = 0.002$  respectively]. BACE1 Tg mice had PGF weights that were not significantly affected by genotype  $F(1,21) = 0.08$ ,  $p = 0.29$  but almost significantly affected by gender  $F(1,21) = 3.91$ ,  $p$



= 0.06 (Figure 5.3B). No gender x genotype interaction was seen for either line of mice. The BACE1 KO mice had 63% less and BACE1 Tg mice 5% less PGF than respective wt controls.

### 5.3.3.2 Brown Adipose Tissue



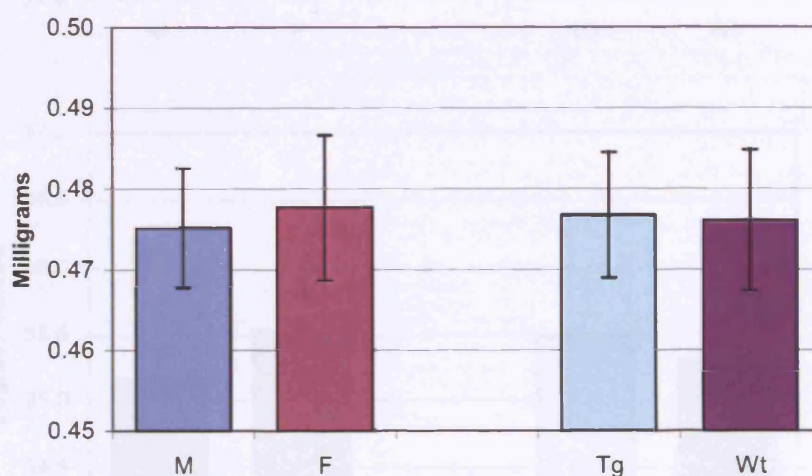
**Figure 5.4 BACE1 Brown Adipose Tissue Weight**

A) KO mice. B) Tg mice. N = 7 M KO, 8 F KO, 12 M wt, 9 F wt controls. N = 5 M Tg, 6 F Tg, 7 M wt, 7 F wt controls. Data are represented by gender or by genotype. Mean  $\pm$  SEM. \*  $p < 0.05$ . \*\*  $p < 0.01$

Analysis of the Brown Adipose Tissue (BAT) weight of BACE1 KO mice revealed a significant effect of gender  $F(1,12) = 6.46$ ,  $p = 0.026$  but no effect of genotype  $F(1,12) = 3.13$ ,  $p = 0.10$  (Figure 5.4A). Analysis of the BACE1 Tg mouse BAT weight revealed a significant effect of gender  $F(1,12) = 11.5$ ,  $p = 0.005$  but no

effect of genotype  $F(1,12) = 1.30$ ,  $p = 0.28$  (Figure 5.4B). No gender x genotype interaction was seen for either line of mice. The BACE1 KO mice had 28% less and BACE1 Tg mice 27% more BAT than respective wt controls. Regardless of genotype, males from the BACE1 KO line of mice had 62% more BAT than females. Similarly males from the BACE1 Tg line of mice had 110% more BAT than females.

### 5.3.3.3 Brain Weight



**Figure 5.5 BACE1 Tg mice Brain Weight**

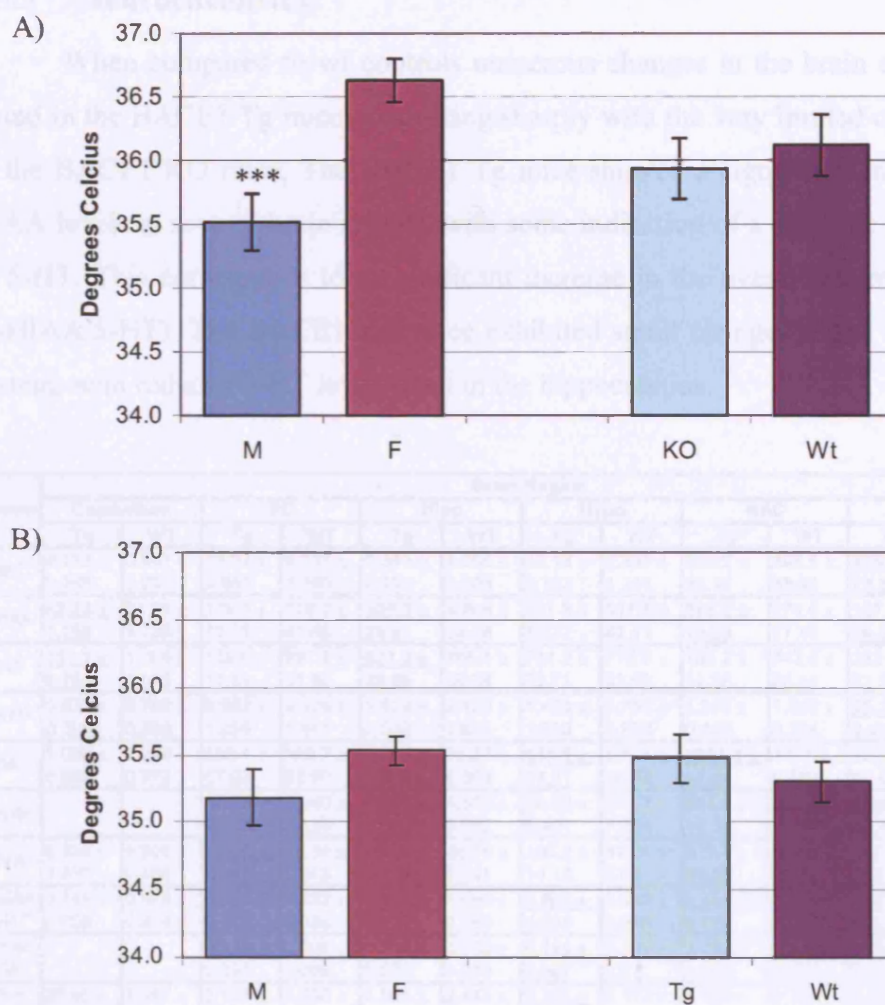
N = 5 M Tg, 6 F Tg, 7 M wt, 7 F wt controls. Data are represented by gender or by genotype. Mean  $\pm$  SEM.

Analysis of the Brain weight of the BACE1 Tg mice revealed no significant effect of gender  $F(1,12) = 0.041$ ,  $p = 0.84$  or genotype  $F(1,12) = 0.003$ ,  $p = 0.96$ . No gender x genotype interaction was seen (Figure 5.5). The BACE1 Tg mouse brains were 0.1% heavier than wt controls.

### 5.3.4 Body Temperature

No significant effect of genotype on temperature was found however an effect of gender was seen. Males were 3% (KO mouse line) or 1% (Tg mouse line) cooler than females.





**Figure 5.6 BACE1 Body Temperatures**

**A) KO mice. B) Tg mice.** N = 7 M KO, 8 F KO, 12 M wt, 9 F wt controls. N = 5 M Tg, 6 F Tg, 7 M wt, 7 F wt controls. Data are represented by gender or by genotype. Mean  $\pm$  SEM. \*\*\*  $p < 0.001$ .

Analysis of the Body Temperature of the BACE1 KO mice revealed a significant effect of gender  $F(1,32) = 14.6$ ,  $p < 0.001$  but no effect of genotype  $F(1,32) = 1.09$ ,  $p = 0.31$  (Figure 5.6A). A planned comparison of temperature revealed no significant difference between male KO and male wt controls  $F(1,32) = 0.25$ ,  $p = 0.62$  or between female KO and female wt controls  $F(1,32) = 0.94$ ,  $p = 0.34$ . Analysis of the BACE1 Tg mice Body Temperature revealed a near significant effect of gender  $F(1,16) = 3.58$ ,  $p = 0.07$  but no effect of genotype  $F(1,16) = 0.33$ ,  $p = 0.57$  (Figure 5.6B). No gender  $\times$  genotype interaction was seen for either line of mice. Males from the BACE1 KO line of mice were  $1.1^{\circ}\text{C}$  (3%) cooler than females. Males from the BACE1 Tg line of mice were  $0.35^{\circ}\text{C}$  (1%) cooler than females.

### 5.3.5 Neurochemistry

When compared to wt controls numerous changes in the brain amines were found in the BACE1 Tg mice contrasting sharply with the very limited changes seen in the BACE1 KO mice. The BACE1 Tg mice showed a significant increase in 5-HIAA levels in several brain regions with some indication of a decrease in the levels of 5-HT. This corresponds to a significant increase in the overall turnover of 5-HT (5-HIAA/5-HT). The BACE1 KO mice exhibited small changes in the serotonergic system, with reduced 5-HT levels seen in the hippocampus.

A)

Amine	Brain Region											
	Cerebellum		FC		Hipp		Hypo		NAC		Striatum	
	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT
3MT	0.518 ± 0.268	0.947 ± 0.292	13.07 ± 3.683	6.237 ± 1.150	1.343 ± 0.192	1.355 ± 0.405	52.13 ± 0.762	2.537 ± 1.214	330.2 ± 38.17	395.5 ± 33.96	1151.6 ± 72.21	870.6 ± 89.59
5HIAA	82.23 ± 7.152	57.87 ± 5.739	179.3 ± 10.05	176.2 ± 41.66	508.5 ± 38.97	408.6 ± 24.28	481.5 ± 66.72	311.8 ± 42.11	395.0 ± 19.28	274.6 ± 17.10	157.5 ± 16.05	108.9 ± 6.636
5HT	111.1 ± 6.783	101.0 ± 10.05	538.9 ± 11.84	597.7 ± 51.89	627.2 ± 20.69	705.1 ± 36.34	781.2 ± 70.71	778.5 ± 53.06	793.2 ± 12.38	743.0 ± 25.48	262.1 ± 22.50	263.1 ± 22.69
5HTP	0.637 ± 0.328	0.782 ± 0.269	3.992 ± 1.255	4.128 ± 1.441	1.922 ± 0.532	2.120 ± 0.626	5.453 ± 1.530	7.753 ± 3.906	1.285 ± 0.409	1.510 ± 0.294	25.30 ± 6.079	9.990 ± 3.359
DA	1.008 ± 0.689	1.249 ± 0.372	136.1 ± 57.92	125.7 ± 92.90	7.267 ± 1.546	20.27 ± 8.363	354.5 ± 37.21	500.6 ± 54.72	1021.8 ± 17.91	1077.9 ± 9.583	9820.8 ± 87.85	9812.6 ± 93.33
Dopac			27.56 ± 4.333	14.40 ± 7.133	1.280 ± 0.363	4.312 ± 0.532	50.83 ± 5.401	51.07 ± 7.360	694.8 ± 15.54	711.2 ± 12.37	1600.6 ± 83.86	1149.7 ± 76.00
HVA	5.330 ± 1.327	4.505 ± 0.888	136.8 ± 6.967	76.34 ± 9.612	25.05 ± 1.397	26.76 ± 2.341	100.2 ± 14.12	84.04 ± 5.60	912.4 ± 68.09	787.3 ± 76.86	1974.5 ± 116.6	1461.5 ± 174.3
5-HIAA /5-HT	0.745 ± 0.059	0.576 ± 0.025	0.332 ± 0.014	0.282 ± 0.036	0.819 ± 0.081	0.590 ± 0.055	0.602 ± 0.035	0.393 ± 0.036	0.498 ± 0.024	0.372 ± 0.028	0.620 ± 0.073	0.423 ± 0.029
Dopac /DA			0.370 ± 0.097	0.205 ± 0.065	0.212 ± 0.074	0.338 ± 0.080	0.144 ± 0.007	0.103 ± 0.011	0.680 ± 0.006	0.660 ± 0.008	0.163 ± 0.008	0.117 ± 0.007
HVA /DA	35.90 ± 24.37	4.647 ± 0.934	2.157 ± 0.605	1.890 ± 0.449	4.505 ± 1.300	2.443 ± 0.709	0.282 ± 0.031	0.175 ± 0.009	0.889 ± 0.053	0.730 ± 0.070	0.201 ± 0.011	0.148 ± 0.017
Dopac /HVA			0.198 ± 0.024	0.170 ± 0.068	0.053 ± 0.017	0.165 ± 0.025	0.534 ± 0.054	0.586 ± 0.050	0.777 ± 0.042	0.956 ± 0.111	0.815 ± 0.030	0.856 ± 0.121

B)

Amine	Brain Region											
	Cerebellum		FC		Hipp		Hypo		NAC		Striatum	
	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT
3MT	3.330 ± 0.645	5.334 ± 3.434	3.870 ± 1.235	1.146 ± 0.364	0.973 ± 0.423	0.928 ± 0.556	4.012 ± 1.225	4.307 ± 1.703	103.1 ± 28.09	247.0 ± 84.52	789.4 ± 76.59	868.0 ± 60.85
5HIAA	64.73 ± 7.537	71.14 ± 4.875	149.8 ± 10.66	156.0 ± 7.468	327.1 ± 14.88	368.8 ± 26.51	397.1 ± 14.01	401.8 ± 17.48	247.9 ± 43.88	279.7 ± 30.41	179.5 ± 10.52	169.2 ± 28.56
5HT	115.8 ± 18.07	127.4 ± 10.97	622.6 ± 51.61	640.2 ± 29.78	524.4 ± 25.16	607.8 ± 25.15	873.7 ± 13.70	863.9 ± 14.28	780.8 ± 43.90	762.6 ± 62.08	300.6 ± 24.35	354.7 ± 32.09
5HTP			1.910 ± 0.587	2.830 ± 1.539	0.363 ± 0.101	0.527 ± 0.194	2.965 ± 0.907	3.898 ± 1.100	11.59 ± 1.328	11.08 ± 3.576	6.588 ± 3.511	4.103 ± 1.438
DA			25.58 ± 3.401	33.33 ± 4.52	8.419 ± 3.366	7.981 ± 0.861	469.0 ± 39.67	459.9 ± 50.85	6875.2 ± 419.2	7623.1 ± 479.9	9569.8 ± 67.9	9953.2 ± 52.6
Dopac			24.35 ± 3.324	24.66 ± 3.049	2.715 ± 0.508	4.693 ± 1.138	73.61 ± 5.826	72.37 ± 5.065	626.1 ± 41.22	793.1 ± 101.6	972.7 ± 58.97	1162.6 ± 115.0
HVA			79.46 ± 11.52	79.86 ± 6.672	28.75 ± 3.353	26.34 ± 2.785	143.4 ± 6.849	124.1 ± 12.19	931.4 ± 65.87	917.5 ± 61.12	1616.3 ± 92.33	1628.9 ± 114.8
5-HIAA /5-HT	0.573 ± 0.038	0.568 ± 0.041	0.244 ± 0.012	0.245 ± 0.012	0.626 ± 0.019	0.606 ± 0.032	0.455 ± 0.019	0.466 ± 0.022	0.309 ± 0.045	0.375 ± 0.049	0.616 ± 0.054	0.488 ± 0.078
Dopac /DA			1.075 ± 0.222	0.781 ± 0.129	0.630 ± 0.225	0.601 ± 0.134	0.162 ± 0.018	0.161 ± 0.01	0.092 ± 0.006	0.104 ± 0.01	0.101 ± 0.005	0.117 ± 0.011
HVA /DA			3.533 ± 0.787	2.558 ± 0.365	6.574 ± 1.964	3.404 ± 0.324	0.324 ± 0.046	0.287 ± 0.044	0.138 ± 0.012	0.121 ± 0.005	0.169 ± 0.01	0.164 ± 0.011
Dopac /HVA			0.310 ± 0.015	0.308 ± 0.028	0.093 ± 0.011	0.173 ± 0.028	0.522 ± 0.057	0.615 ± 0.077	0.683 ± 0.054	0.866 ± 0.089	0.612 ± 0.051	0.723 ± 0.070

Table 5.2 BACE1 Neurochemistry

A) Tg mice B) KO mice. Significant differences are in bold and underlined. Measures are in ng/g of wet tissue.

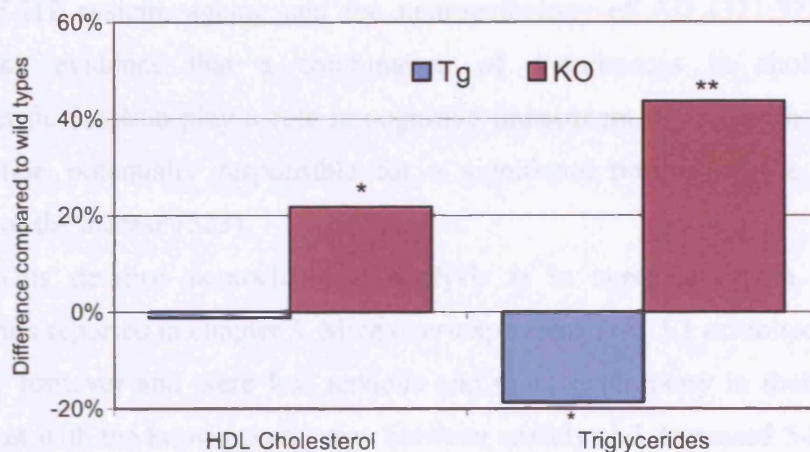
BACE1 Tg mice (Table 5.2B) showed a significant increase in 5-HT turnover in cerebellum  $F(1,10) = 6.87$ ;  $p = 0.02$ , hippocampus  $F(1,10) = 5.45$ ;  $p = 0.04$ , hypothalamus  $F(1,9) = 13.6$ ;  $p = 0.005$ , nucleus accumbens  $F(1,10) = 12.0$ ;  $p = 0.006$  and caudate striatum  $F(1,10) = 6.31$ ;  $p = 0.03$  compared to wt controls. Concurrently, this corresponded to a significant increase in the level of 5-HIAA in cerebellum  $F(1,10) = 7.06$ ;  $p = 0.02$ , hippocampus  $F(1,10) = 4.74$ ;  $p = 0.054$ , nucleus accumbens  $F(1,10) = 21.9$ ;  $p < 0.001$  and striatum  $F(1,10) = 7.82$ ;  $p = 0.019$ . In addition, a significant decrease was observed in the level of dopamine in the hypothalamus  $F(1,9) = 5.16$ ;  $p = 0.05$  and nucleus accumbens  $F(1,10) = 7.61$ ;  $p = 0.02$ . HVA levels were significantly increased in the frontal cortex  $F(1,10) = 25.9$ ;  $p < 0.001$  and striatum  $F(1,10) = 5.98$ ;  $p = 0.034$  of Tg mice, with a significant decrease of DOPAC observed in the hippocampus  $F(1,10) = 22.2$ ;  $p < 0.001$  and a significant increase in DOPAC in the striatum  $F(1,10) = 15.9$ ;  $p = 0.003$ . These changes resulted in an overall significant increase in dopamine turnover (assessed by calculating DOPAC/DA ratios) in the hypothalamus  $F(1,9) = 10.4$ ;  $p = 0.01$  and striatum  $F(1,10) = 17.9$ ;  $p = 0.002$ , with a trend towards an increase in the nucleus accumbens  $F(1,10) = 3.92$ ;  $p = 0.076$ . Furthermore, a significant increase in dopamine turnover (HVA/DA) was observed in the hypothalamus  $F(1,9) = 9.04$ ;  $p = 0.015$  and striatum  $F(1,10) = 6.57$ ;  $p = 0.028$  and a significant decrease in dopamine turnover (DOPAC/HVA) in the hippocampus  $F(1,10) = 14.2$ ;  $p = 0.004$ .

Analysis of data from the BACE1 KO mice (Table 5.2A) revealed three significant changes in neurochemistry. These were a decrease in 5-HT levels in the hippocampus  $F(1,10) = 5.49$ ,  $p = 0.04$ , a decrease in dopamine turnover (DOPAC/HVA) in the hippocampus  $F(1,10) = 6.88$ ,  $p = 0.026$  and a decrease in total dopamine content in the striatum  $F(1,10) = 19.9$ ;  $p = 0.001$  when compared to wt controls.

### 5.3.6 Blood Biochemistry

BACE1 KO mice had significantly elevated levels of circulating High Density Lipoprotein (HDL) Cholesterol and Triglycerides while BACE1 Tg mice had significantly lowered circulating Triglyceride levels.





**Figure 5.7 BACE1 Blood lipids**

Percent difference compared to wt controls. N=10 for each gender and genotype.

\* $p < 0.05$ , \*\* $p < 0.01$ .

Analysis of the Blood Biochemistry of the young mice revealed a significant increase in HDL Cholesterol  $F(1,19) = 4.66$ ,  $p = 0.044$  and Triglycerides  $F(1,22) = 7.98$ ,  $p = 0.01$  in BACE1 KO mice when compared to controls. A significant decrease in Triglycerides  $F(1,33) = 4.18$ ,  $p = 0.049$  was seen in the BACE1 Tg mice when compared to wt controls. A small, non-significant decrease in HDL Cholesterol was also seen in the BACE1 Tg mice  $F(1,33) = 0.37$ ,  $p = 0.54$ . HDL Cholesterol was 1.2% less (Tg) and 21% more (KO) than respective controls and Triglycerides were 19% less (Tg) and 42% more (KO) than respective wt controls.

## 5.4 Discussion

The young BACE1 Tg mice showed a significant increase in 5-HIAA levels in several brain regions with some indication of a decrease in the levels of 5-HT when compared to wt controls. This corresponds to a significant increase in the overall turnover of 5-HT (5-HIAA/5-HT), which is generally thought to be indicative of an increase in 5-HT release. The young BACE1 KO mice also exhibited small changes in the serotonergic system, with reduced 5-HT levels seen in the hippocampus. These findings correlate with the observed anxiolytic phenotype in the BACE1 Tg animals since 5-HT has been implicated in anxiety (319) and drugs which elevate synaptic 5-HT, such as serotonin specific reuptake inhibitors (SSRI) are clinically effective as anxiolytics (320). Although it is difficult to draw definitive

conclusions from these findings, a link has been demonstrated between disturbances in the 5-HT system, ageing and the neuropathology of AD (321;322). There is increasing evidence that a combination of disturbances in cholinergic and serotonergic function play a role in cognitive impairment in AD, with serotonergic dysfunction potentially responsible for a significant portion of the behavioural aspects of the disease (323).

This detailed neurochemical analysis is in agreement with the anxiety behaviours reported in chapter 3. Mice over-expressing BACE1 exhibited an increase in 5-HT turnover and were less anxious and more exploratory in their behaviour, consistent with the known correlation between anxiety and decreased 5-HT turnover (324). In contrast, mice lacking BACE1 displayed an anxious and less exploratory phenotype.

In addition to the effects of BACE1 overexpression on the serotonergic system, there also appear to be some changes to the dopaminergic system in these animals (e.g. the decreased dopamine in hypothalamus and nucleus accumbens, increased HVA in frontal cortex and striatum). Changes in dopaminergic neurotransmitter systems have also been observed in a number of post-mortem studies of AD brain (322;325).

While the male BACE1 Tg mice put on more weight and were heavier than male wt controls they did not have more peri-genital fat (PGF) but did have more brown adipose tissue (BAT). BAT has a high density of mitochondria and is integral to lipid metabolism and thermogenesis (326). The reduced body weight and inability to put on weight shown by both male and female BACE1 KO mice was reflected in the reduced weight of PGF and trend towards a reduction of BAT in these mice.

This correlation between body weight and the presence or absence of the BACE1 gene indicates a role for BACE1 in metabolism. There is also a gender specific difference indicating a hormonal component to this effect. Changes in BACE1 expression have been reported in AD patients. These reports are associated with increased BACE1 enzyme levels in human post mortem AD brains (309;327) as well as in aged monkey, mouse and non-demented human brains (328). Additionally BACE1 expression in sporadic AD patients was shown to be increased with elevated  $\beta$ -amyloid peptide load (329). The suggested mechanism for this increase in BACE1 expression is that an unknown process stabilises BACE1 mRNA long enough for additional enzyme production. This elevation of BACE1 expression may be expected

in AD patients as, if the expression is translated into enzyme activity, increased cleavage of APP at the  $\beta$ -secretase cleavage sites will result in elevated production of the  $\beta$ -amyloid found in AD patients. Decreased BACE1 expression has not been investigated in the same way as, once BACE1 was identified as  $\beta$ -secretase, the main thrust of research centred on the activity of BACE1 as  $\beta$ -secretase and its involvement in  $\beta$ -amyloid production.

The reduction in weight shown by the BACE1 KO mice may be accounted for by reduced adipose tissue deposition, which does not result from increased physical activity or heat production. One explanation for the reduced adipose tissue is a reduction in food intake. Appetite is controlled in part by the neuroendocrine system of the Hypothalamic Pituitary Axis (HPA) (for a review see (330)). One potent anorectic agent in this system of control is  $\alpha$ -Melanocyte Secreting Hormone ( $\alpha$ -MSH).  $\alpha$ -MSH is secreted from the Arcuate Nucleus of the hypothalamus in the CNS (for a review see (331)). In addition to this function  $\alpha$ -MSH has is involved in skin pigmentation and has been shown to have a role in energy homeostasis and other physiological functions (332). Soluble APP $\alpha$  (sAPP $\alpha$ ) has an identical effect on melanocytes as  $\alpha$ -MSH in the periphery, stimulating melanin production and pseudopodial extension in melanocytes (333). When  $\beta$ -secretase activity is removed in BACE1 KO mice the amount of APP substrate available for  $\alpha$ -secretase cleavage is increased. If an interaction between sAPP $\alpha$  and  $\alpha$ -MSH can be established then the effect of removing BACE1 expression on weight may be explained.

The weight loss shown by the BACE1 KO mice is positively correlated with the decreased levels of serotonin seen in the hippocampus of these mice. A number of studies have shown that serotonin appears to be involved in the Neuropeptide Y network of food intake regulation (334-336), pointing towards a potential link between the decreased levels of serotonin and the weight loss seen in the BACE1 KO mice. In humans, anorexia is seen during aging at the time of life when decreased serotonin signalling is also observed (337). AD patients show weight loss during the disease progression (338) and the serotonergic system is known to be disturbed in these patients with a variable decrease in the serotonin content of several brain regions (339).

Young BACE1 KO mice have elevated circulating HDL Cholesterol and Triglyceride levels while young BACE1 Tg mice have depleted circulating

Triglyceride levels. These changes in blood serum biochemistry may result from altered lipid metabolism with decreased insulin production leading fat breakdown (lipolysis) and elevated serum triglyceride levels in KO mice and increased insulin production and fat storage (lipogenesis) and triglyceride levels in Tg mice. This could explain the reduction in adipose tissue in KO mice and increased adipose tissue seen in male Tg mice.

The presence of a copper binding site in the BACE1 protein may indicate a role for BACE1 as a mediator of weight via copper binding (104) as rats deprived of dietary copper lose weight (340). In addition other explanations for this decrease in weight shown by BACE1 KO mice may be revealed with further studies targeted at dietary and metabolic effects of BACE1 removal.

# *Chapter 6 - Discussion and Conclusions*



The results from chapter 1 indicate that both BACE1 Tg and BACE1 KO mouse lines were essentially unchanged from wild type littermate controls with some subtle differences in behaviour. The slightly lower body weight and reduced activity in the test arena of the BACE1 Tg mice plus the slightly firmer limb tone and improved behaviour of the BACE1 KO mice in the wire manoeuvre test, did not affect overall locomotion of either line. The increased faecal production, seen in the KO mice, has been reported to correlate with increased emotion and anxiety (286). Furthermore, the decreased exploratory activity of the BACE1 KO mice in the holeboard test is also suggestive of an anxious behavioural phenotype. In contrast to these findings, the BACE1 Tg mice displayed a less anxious behaviour in the holeboard test with significantly more exploration. This behaviour is typical of that seen following administration of anxiolytic drugs to wild type mice (287). Overall, the BACE1 KO and higher expressing BACE1 Tg mice present broadly opposite phenotypes with respect to faecal pellet number and holeboard activity. The lower expressing BACE1 Tg mouse line represented an intermediate phenotype between these two mouse lines. These results indicate a bold, more exploratory behaviour in the BACE1 Tg animals with a higher level of BACE1 expression and a timid, less exploratory behaviour in the BACE1 KO animals compared to controls.

The BACTAS double Tg mice had a gender specific difference in weight with double Tg females being 29% lighter than wild type females. Interestingly BACTAS mice were shorter than control mice and females were shorter than males though this did not have a significant effect on their weight. The increased arena activity and tremor shown by the BACTAS mice may be indicative of a poor state of health compared to wild type control mice. This may also be reflected in the reduced likelihood of biting when provoked and the reduced aggression shown by the BACTAS mice. These mice were well advanced in plaque pathology (data not shown) and it is possible that they had started to exhibit some of the phenotypes of increased locomotion (288) and passivity (289) sometimes seen in AD patients.

Analysis of anxiety and cognition of the BACE1 Tg and KO mice revealed several differences compared to wild type controls. When BACE1 Tg mice were examined in classic tests for anxiety, the open field test and elevated plus maze test, they were not found to be significantly different from wild type controls. They did spend 26% more time in the open arms of the elevated plus mice and had approximately 60% fewer incidents of 'stretch attend' behaviour, an indicator of

increased anxiety. These results, in combination those from chapter 2, indicate a reduced anxiety phenotype for the Tg mice. In identical tests the BACE1 KO mice travelled almost 40% less distance and spent just under 50% less time in the inner zone of an open field compared to controls indicating an anxious phenotype. These mice also exhibited almost double the number of grooming bouts and showed a trend for less time on the open arms of the elevated plus maze when compared to wild type controls. Taken together these results indicate an increased anxiety phenotype in the BACE1 KO mice. The broad phenotype of the two different lines of BACE1 mice is an increased anxiety in the KO mice and a reduced anxiety in the Tg mice.

When tested for differences in peri-rhinal cortex dependent cognitive function in the object recognition test no impairment was seen for BACE1 Tg or KO mice compared to wild type controls. The fear conditioning test examined hippocampus and amygdala dependent cognitive function. BACE1 Tg mice were unimpaired in hippocampal or amygdala related function in this test. Conversely BACE1 KO mice showed a gender specific impairment with male KO mice having impaired hippocampal and amygdala function while female KO mice were only impaired in hippocampal function when measured in this test.

BACE1 cleavage of APP is hypothesised to be the rate limiting step in the production of  $\beta$ -amyloid peptide. Thus elevating human BACE1 rather than PS1<sub>M146V</sub> expression, in conjunction with human APP<sub>KM670/671NL</sub> in mouse models might be expected to accelerate the cognitive impairment known to be associated with increased  $\beta$ -amyloid production (263). The spatial learning and memory of two double Tg lines of mice, BACTAS and TASTPM, were examined in the watermaze test. The BACTAS mice were unimpaired in learning and memory or in synaptic plasticity in this test. However this sparing of synaptic function in the BACTAS mice was not seen in the TASTPM mice. These mice had impairment in learning and in the accurate retention of a learned platform position when compared to wild type. The impaired cognition seen in the TASTPM mice was not reflected in the BACTAS mice suggesting that increased mutant PS1 expression rather than increased BACE1 expression accelerates the decrease in cognitive performance.

Recently Rockenstein *et al* (341) have demonstrated that high levels of BACE1 activity are sufficient to elicit neurodegeneration and neurological decline *in vivo*. The authors present data to show impaired watermaze performance in hBACE1 Tg, mutant hAPP751 Tg and mutant hAPP751 x hBACE1 dTg mouse lines. These

mice were on a mixed background strain and were tested at 6 months of age. This data is not in agreement with data presented in this thesis on the BACTAS dTg mouse line. Differences in background mouse strains (congenic v hybrid), the levels of BACE1 Tg expression (reported to be high in the paper) and promoter used (Thy1 for both lines in the publication v Cam Kinase II-BACE1 & Thy1-APP<sub>KM670/671NL</sub> in this thesis) may account for this discrepancy. The authors do state that they saw decreased levels of A $\beta$ <sub>1-x</sub> and A $\beta$ <sub>1-42</sub> in the hBACE1/hAPP mice in agreement with one publication but in disagreement with several others.

The lack of cognitive impairment seen when human BACE1 expression is increased on its own, or in conjunction with human APP<sub>KM670/671NL</sub> in the mouse models described in this thesis, suggests that BACE1 is less important than PS1 in the progress of cognitive decline. Removing endogenous BACE1 led to a sex specific impairment in hippocampal and amygdala related memory function. The amygdala is involved in emotional memory as well as anxiety, thus the anxious phenotype observed when endogenous BACE1 is removed may relate to the impaired memory function seen in the fear conditioning test. Clearly BACE1 has other functions distinct from its activity as  $\beta$ -secretase. These data demonstrating subtly impaired cognitive function in BACE1 KO mice is in agreement with a recent publication demonstrating impaired cognition in another line of BACE1 KO mice (263). Further work is required to explore the nature of these behavioural phenotypes and how they may be reflected in the human population.

Specific anxiety and cognitive related effects have been found with the removal of endogenous BACE1. While any therapeutic agent would only reduce BACE1 enzyme activity and not remove the protein entirely, the high homology between BACE1 and BACE2 may mean that a reduction in BACE2 activity as a consequence of BACE1 inhibition must also be considered. To address this issue a single line of mice was produced with both BACE1 and BACE2 endogenous gene function removed. Additionally while BACE1 has been shown to be  $\beta$ -secretase and mice lacking BACE1 are physiologically normal, pharmacological inhibition of BACE1 activity alone as a treatment for AD may be ineffective if BACE2 activity could substitute for the lack of BACE1. Under these circumstances BACE inhibitors would need to inhibit both BACE1 and BACE2.

The BACE1/2 double KO mice showed subtle, gender specific differences in anxiety and motor activity and co-ordination compared to wild type controls. The trend for increased anxiety seen in the male BACE1/2 double KO mice was similar to that seen in male BACE1 KO mice. The additional deletion of the BACE2 gene did not appear to increase the anxiety in these mice. Learning in these double KO mice was unimpaired however memory of a specific target, namely the platform location to which the mice were trained, was poorer in double KO mice compared to wild type controls. Interestingly this impairment in memory was not sex specific but was present in the same neuronal substrate, the hippocampus, as that found in the BACE1 KO mice in the fear conditioning test.

Both male and female BACE1/2 double KO mice were lighter than respective wild type controls. The improved motor co-ordination and decreased home cage inactivity shown by female double KO mice when compared to wild type controls demonstrate that though they were lighter than controls they had a more robust motor function and were more active. A more extensive examination of body fat and muscle composition may reveal a fuller explanation of this result. No differences in gross pathology were seen. Overall very few differences were seen in the BACE1/2 double KO mice compared to controls beyond those observed in BACE1 KO mice.

In keeping with data reported in chapters 2 and 3 of this thesis and with previously published work (310), these results might suggest that inhibition of both BACE1 and BACE2 would lead to anxiety related behaviour in treated patients. However, one key difference is that here mice have been generated which completely lack BACE1 and BACE2 proteins while the administration of a BACE inhibitor as a therapeutic would not change protein levels, at least in the short term, only inhibit the enzyme activity.

Young BACE1 Tg mice showed an increase in 5-HT turnover. Young BACE1 KO mice also exhibited small changes in the serotonergic system, with reduced 5-HT levels seen in the hippocampus. There is increasing evidence that a combination of disturbances in cholinergic and serotonergic function play a role in cognitive impairment in AD, with serotonergic dysfunction potentially responsible for a significant portion of the behavioural aspects of the disease (323). This detailed neurochemical analysis in chapter 5 is in agreement with the anxiety behaviours reported in chapter 3. Mice over-expressing BACE1 exhibited an increase in 5-HT turnover and were less anxious and more exploratory in their behaviour, consistent

with the known correlation between anxiety and decreased 5-HT turnover (324). In contrast, mice lacking BACE1 displayed an anxious and less exploratory phenotype. In addition to the effects of BACE1 overexpression on the serotonergic system, there also appeared to be some changes to the dopaminergic system in these animals. Changes in dopaminergic neurotransmitter systems have been observed in a number of post-mortem studies of AD brain (322;325). These results extend our current knowledge of BACE1 mouse models and reveal an unexpected link between BACE1, serotonergic neurotransmission and behaviour.

Male BACE1 Tg mice put on more weight and were heavier than male wild type controls having more brown adipose tissue (BAT). The reduced body weight and inability to put on weight shown by both male and female BACE1 KO mice was reflected in the reduced weight of peri-genital fat (PGF) and trend towards a reduction of BAT in these mice. This correlation between body weight and the presence or absence of the BACE1 gene indicates a role for BACE1 in metabolism. The reduction in weight shown by the BACE1 KO mice is accounted for by reduced adipose tissue deposition that does not result from increased heat production or differences in motor ability. One explanation for the reduced adipose tissue may be a reduction in food intake. Appetite is controlled in part by the neuroendocrine system of the Hypothalamic Pituitary Axis (HPA) and several potent anorectic agents in this system could be altered in BACE1 KO mice.  $\alpha$ -Melanocyte Secreting Hormone ( $\alpha$ -MSH) has been shown to have a role in energy homeostasis and other physiological functions and has the same effect as soluble APP $\alpha$  (sAPP $\alpha$ ) on melanocytes as  $\alpha$ -MSH in the periphery. When  $\beta$ -secretase activity is removed in BACE1 KO mice the amount of APP substrate available for  $\alpha$ -secretase cleavage is increased. If an interaction between sAPP $\alpha$  and  $\alpha$ -MSH can be established then the effect of removing BACE1 expression on weight may be explained. Another hormone found that may explain the phenotype of the BACE1 KO mice is corticotrophin releasing hormone (CRH). This hormone acts with arginine vasopressin (AVP) to stimulate the release of adrenocorticotrophic hormone (ACTH) from the pituitary. CRH is known to be elevated in corticotrophin releasing hormone binding protein (CRH-BP) KO mice and this increase in 'free' CRH is the most likely explanation for the anorectic and anxiogenic phenotype seen in these mice (342), a phenotype very similar to that seen in the BACE1 KO mice.

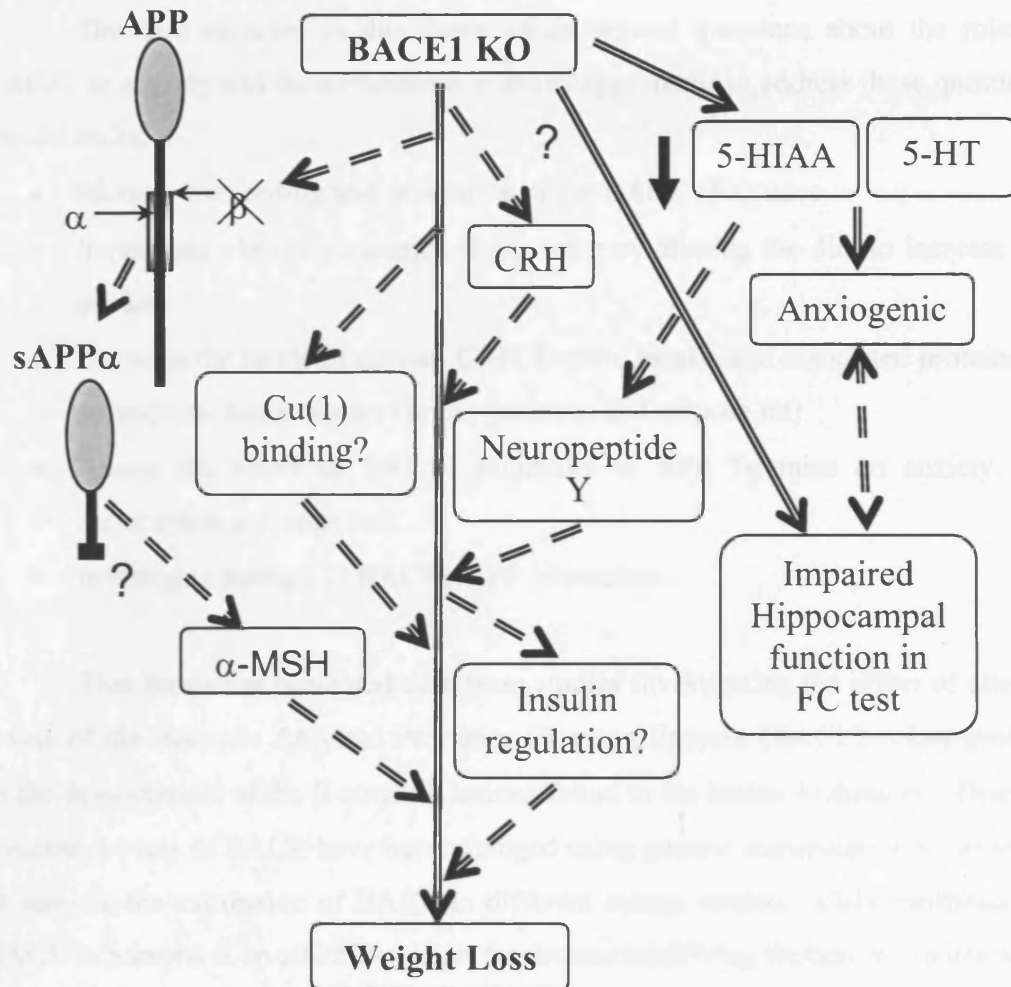


Young BACE1 KO mice have elevated circulating HDL Cholesterol and Triglyceride levels while young BACE1 Tg mice have depleted circulating Triglyceride levels. If lipolysis and lipogenesis do account for these changes then insulin metabolism may also be changed in these mice. Interestingly while no direct correlation between insulin and BACE1 have been shown, BACE1 is expressed in the same cellular type as insulin in the pancreas, the  $\beta$ -cells, though the protein is not expressed as an active enzyme. If an interaction between these proteins can be identified a potential link between BACE1 expression and weight control, possibly via insulin and leptin regulation, may be found. In a recent paper Steen *et al* (343) suggested that the disparate symptoms and cellular pathologies of sporadic AD may have a common cause in the reduced glucose utilisation and deficient energy metabolism seen in the early phase of the disease. The paper describes extensive abnormalities in insulin and insulin-like growth factor signalling mechanisms in the brains of AD patients. The authors then goes on to suggest that these findings indicate a role for impaired insulin signalling in the pathogenesis of AD and propose the term “Type 3 Diabetes” to describe AD. The findings described in this thesis also implicate BACE1 in fat metabolism and show that BACE1 KO mice have altered cholesterol and triglyceride levels.

The presence of a copper binding site in the BACE1 protein may indicate a role for BACE1 as a mediator of weight via copper binding as rats deprived of dietary copper lose weight. In addition other explanations for this decrease in weight shown by BACE1 KO mice may be revealed with further studies targeted at dietary and metabolic effects of BACE1 removal.

There are numerous potential reasons to explain the key findings in this thesis and I present an hypothesis in an attempt to bring together the various potential explanations for these results.

### A BACE-less Hypothesis



A BACE-less hypothesis must incorporate the removal of BACE1 causing increased anxiety and reduced weight in KO mice. Changes in the serotonergic system are known to influence anxiety, hence decreased serotonin at the synapse may account for the anxiogenic phenotype. While the impaired hippocampal function seen in the fear conditioning test by BACE1 KO mice and in the watermaze by the BACE1/2 double KO mice indicates a cognitive role for BACE in the hippocampus this may interact with the anxiogenic phenotype of the KO mice as the cognitive paradigms where an effect was seen all include an element of stress in the test. An explanation of the reduced weight loss does not appear to lie in differences activity or thermogenesis, suggesting appetite may play a role. Differences in appetite may be linked to non -  $\beta$ -secretase activity of BACE1 either in binding copper, HPA

activation via CRH or  $\alpha$ MSH, or by influencing fat metabolism through leptin or insulin regulation.

The data reported in this thesis raises several questions about the role of BACE1 in anxiety and fat metabolism. Future experiments to address these questions should include:

- Monitor the feeding and defecation of the BACE1 KO mice.
- Investigate obesity resistance in the mice by altering the diet to increase fat intake.
- Examine the levels of copper, CRH, Leptin, Insulin and associated proteins in appropriate target tissues (brain, pancreas and adipose fat).
- Assess the effect of BACE1 inhibitors in APP Tg mice on anxiety, fat metabolism and cognition.
- Investigate biology of BACE1/APP interaction.

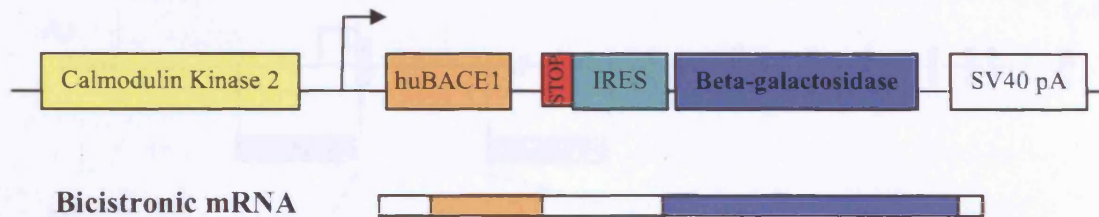
This thesis has presented data from studies investigating the effect of altered levels of the Beta-site Amyloid Precursor Cleaving Enzyme (BACE), a key protein in the development of the  $\beta$ -amyloid lesions found in the brains Alzheimer's Disease patients. Levels of BACE have been changed using genetic manipulation to increase or remove the expression of BACE in different mouse models. While inhibition of BACE in humans is an attractive target for disease modifying therapy in Alzheimer's Disease, differences in anxiety, cognition and metabolism have been found in BACE1 KO mice. Importantly BACE1 inhibition in humans will only seek to reduce the level of enzyme activity to mediate  $\beta$ -amyloid production, whereas in BACE1 KO mice the protein is completely removed. What is clear from the data is the identification of a potential novel role for BACE1 in neurotransmission, behaviour and fat metabolism. In conclusion, although it is presently unclear how BACE1 over- or under-expression results in such changes in neurotransmitter levels and turnover, these neurotransmitter systems are affected in the dementia patients and as such point to an unexpected and interesting role for BACE1 that warrants further investigation. It is hoped that future work will help determine whether these changes are related to the activity of BACE1 as an APP processing enzyme or are in fact indicative of a currently unknown function for this protein.

## *Chapter 7 - Appendices*

## 7.1 *Generating Transgenic and Knockout mice*

### 7.1.1 Expression cassette cloning strategy

The manipulated DNA must have certain elements in order to be expressed successfully in the host genome. At the very basic level a transgene requires a gene of interest and polyadenylation signal driven by a suitable promoter. In order to follow the expression pattern of the transgene at a gross tissue level a reporter gene can be included, separated from the transgene by an IRES (Internal Ribosomal Entry Sequence Element). This allows two gene products to be produced from one promoter thus ensuring that the reporter gene will accurately report where the transgene has been expressed (see figure below).

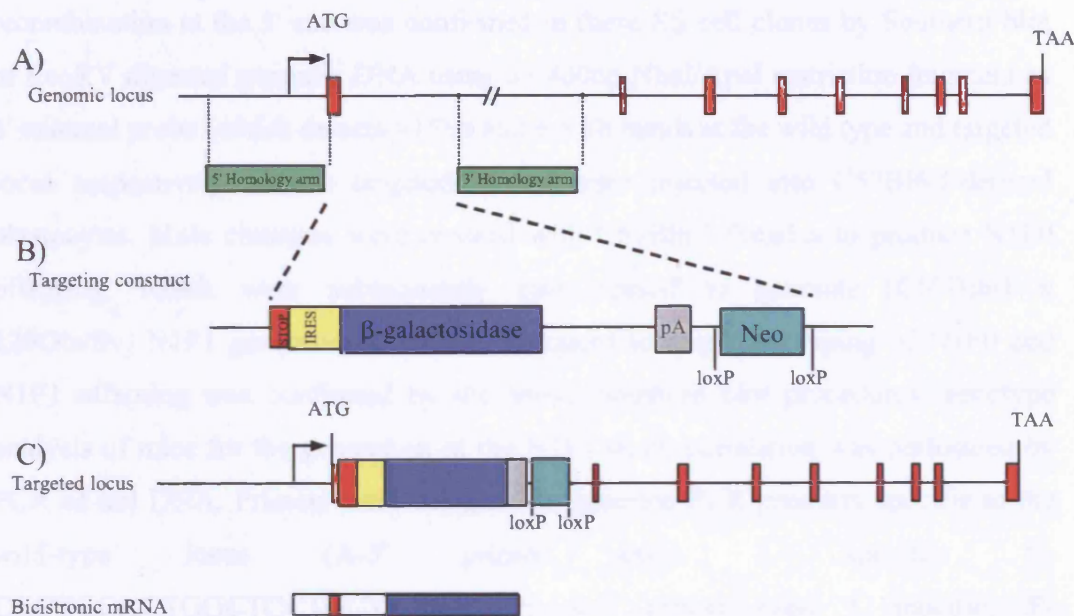


Transgenic mice were generated using an over-expression cassette consisting of the promoter of the  $\alpha$  subunit of the  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase II gene (CaMKII $\alpha$ ) driving expression of the human BACE1 cDNA linked to a LacZ reporter gene via an IRES element. The CaMKII $\alpha$  promoter has been shown to drive neuronal specific expression of transgenes predominantly in the cortex and hippocampus with additional expression in forebrain, amygdala and striatum. The gene construct was microinjected into isogenic C57Bl/6 single celled mouse oocytes. Positive founder mice were identified by PCR using primers specific to the human BACE1 cDNA sequence (5' primer: 5'-GGCTACTACGTGGAGATGACCGTGGG-3'; 3' primer: 5'-CCTGGGTGTAGGGCACATACACACCC-3'; thirty cycles of 94°C (30s), 65°C (30s), and 72°C (60s) giving a product size of 190 bp) and numbers were expanded over two generations to produce experimental animals.



### 7.1.2 BACE1 Knockout Targeting Strategy

When engineering the target gene the most important consideration is ensuring the expression of the endogenous gene is completely removed. If even part of a gene is transcribed the resulting partial gene product may be translated into a functional protein and have unknown effects possibly entirely unrelated to its original function. In order to avoid this situation the endogenous locus of the gene of interest must be genetically engineered to prevent any transcription. This is achieved by manipulating the endogenous locus as close to the transcription initiation site (ATG) as possible. This manipulation should remove at least the first exon of the locus and replace it with a reporter gene, LacZ for example. More of the locus can be removed so as to ensure no functional gene product is transcribed.

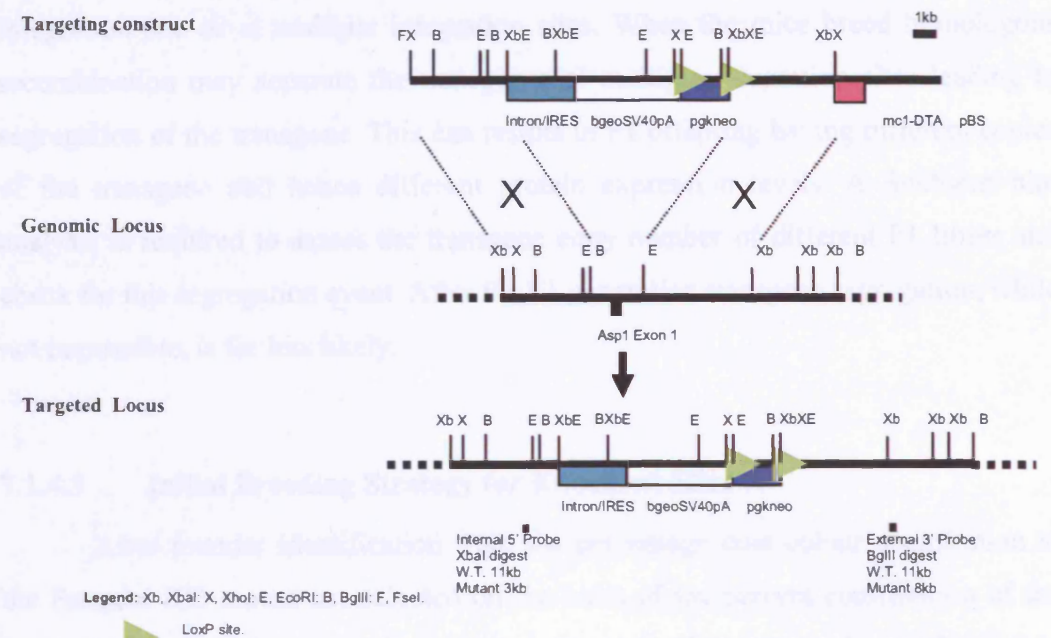


**Structure of a region of the BACE1 genomic locus.** A) Only the first two exons (red boxes) and intron 1 are shown, the rest of the locus remains intact after homologous recombination. The position of the ~300bp *AvrII/XbaI* and ~400bp *NheI/ApaI* restriction fragment probes used to identify homologous recombination events at the 5' and 3' ends respectively are indicated as black boxes. B) Structure of the targeting construct. The 5' and 3' arms of homology are ~2kb *SfoI/Eco47III* and ~4kb *SacI/XbaI* restriction fragments respectively. The *Eco47III* site is 8bp downstream of the BACE1 translation initiation codon. Components of the IRES-LacZ cassette and the PGK-Neo selection cassette are indicated. C) Structure of the targeted locus. Homologous recombination replaces most of exon 1 and ~1kb of intron 1 with the IRES-LacZ cassette and the PGK-Neo selection cassette. Restriction sites: R, EcoRV; Sf, SfoI; E, Eco47III; Sa, SacI; X, XbaI.

Gene targeting was performed in E14.1 embryonic stem (ES) cells, replacing most of the first coding exon of the mouse BACE1 locus with an IRES- LacZ expression cassette and a positive selection cassette containing the neomycin phosphotransferase gene driven by the PGK promoter. 5' and 3' homology arms (~2.0kb SfoI/Eco47III and ~4.0kb SacI/XbaI restriction fragments respectively), were cloned from a 129SVJ genomic BAC library and placed either side of the IRES-LacZ expression cassette and positive selection cassette to generate the targeting construct. Homologous recombination in neomycin resistant ES cells was confirmed by Southern blot of EcoRV digested genomic DNA using a ~300bp AvrII/XbaI restriction fragment as 5' external probe (which detects >15kb and 10.0kb bands at the wild type and targeted locus respectively). Approximately 1 in 100 G418 resistant clones had undergone homologous recombination. Homologous recombination at the 3' end was confirmed in these ES cell clones by Southern blot of EcoRV digested genomic DNA using a ~400bp NheI/ApaI restriction fragment as 3' external probe (which detects >15kb and 6.5 kb bands at the wild type and targeted locus respectively). Three targeted clones were injected into C57Bl6/J-derived blastocysts. Male chimeras were crossed with C57Bl6/J females to produce N1F0 offspring, which were subsequently inter-crossed to generate [C57Bl6/J x 129Ola/Sv] N1F1 generation used in subsequent testing. Genotyping of N1F0 and N1F1 offspring was confirmed by the above Southern blot procedures. genotype analysis of mice for the generation of the N1F1 study population was performed by PCR of tail DNA. Primers were designed to generate PCR products specific to the wild-type locus (A-5' primer exon 1 specific: 5'-CGCTGCACTGGCTCCTGCTATGGG-3'; A-3' primer exon 1 specific: 5'-TCTCCACATAGTAGCCCTGGCCGG-3'; thirty cycles of 94°C (30s), 60°C (30s), and 72°C (60s) were used giving a product size of 220 bp) or targeted locus (N-5', neo gene specific 5' primer: 5'- CCGGCCGCTTGGGTGGAGAGG-3' and N-3', neo gene specific 3' primer: 5'-TCGGCAGGAGCAAGGTGAGATGACA-3'; thirty cycles of 94°C (30s), 68°C (30s), and 72°C (30s) were used giving a product of 299bp).



### 7.1.3 BACE2 Knockout Targeting Strategy



### 7.1.4 Colony Expansion

#### 7.1.4.1 Initial Breeding Strategy for Transgenic Mice.

After identification of the desired mouse line from screening the first, founder, generation of mice screening the colony will need to be expanded in order to generate animals for further characterisation and breeding. Initial breeding regimes will be determined by the properties of the gene of interest, for example sex specificity or copy number (in the case of Tg animals). The rapid expansion of the colony is best achieved by breeding founder male mice to two wild type females. This 'trio' of mice is left in the cage to produce litter after litter until enough F1 generation mice are produced. Gestation in mice is approximately 19-21 days and, when left in the same cage as the male, the female will mate within 12-24 hours of parturition (a post-partum mating) and give birth in another 19-21 days later. This cycle can be repeated successfully up to 6 times before breeding performance is affected.

#### **7.1.4.2 Independently Segregating Transgenes**

When a transgene is directed injected into an embryo multiple copies of the transgene can randomly integrate into the host genome in a concatenation at one integration site or at multiple integration sites. When the mice breed homologous recombination may separate the transgenes at multiple integration sites leading to segregation of the transgene. This can results in F1 offspring having different copies of the transgene and hence different protein expression levels. A Southern blot analysis is required to assess the transgene copy number of different F1 litters and check for this segregation event. After the F1 generation transgene segregation, while not impossible, is far less likely.

#### **7.1.4.3 Initial Breeding Strategy for Knockout Mice**

After founder identification from the percentage coat colour contribution to the Founder KO mouse are selected on the basis of the percent contribution of the injected ES cell to the coat colour. If the host strain of mouse used is C57Bl/6 then the coat colour of the host will be black. If 129 ES Cells are used then the donor coat colour is a light brown. Thus one can assess the percent contribution of the donor ES Cells, and hence the proportion carrying the targeted locus, by looking at coat colour. 100% black mouse will not have incorporated many ES Cells but will breed vigorously whereas a 100% light brown mouse will have incorporated many ES Cells but will not breed well. Five founder mice with more than 50% of their coat colour as light brown should be chosen for breeding onto the F1 generation. The resulting F1 generation mice need to be genotyped to assess whether the targeted locus has transmitted through the germ line successfully. F1 mice carrying the targeted mutation can be interbred at this point to generate mice homozygous for the targeted locus. Assuming they are viable, these mice can be analysed to discover if the locus has been targeted correctly and the protein is indeed knocked out.

#### **7.1.4.4 Background Strain.**

The background strain can have an enormous influence on the phenotype of Tg and KO mice. If isogenic donor single celled embryos are used then over-expression Tg mouse lines will be isogenic from birth. However KO mouse lines will

usually be a mix of C57Bl/6 and 129 strains. In cases where the F1 generation mice is a hybrid mix of strains then more reliable phenotypic data will be obtained from the mouse line being back-crossed to produce an isogenic background. This decision to backcross is made by considering the reasons for making the mouse line. If just one protein is to be investigated in the mouse line then other proteins from the different strains are less likely to have an impact on the data. However if many interacting proteins are to be investigated or behavioural observations made then an isogenic background will produce more reproducible data.

## **7.2 *Materials and Methods – SHIRPA test***

### **7.2.1 Primary SHIRPA test**

Initially the weight of the animals is recorded. Subsequently a number of measures are recorded for each animal when it is placed in a viewing jar, then when it is placed in, on or above an arena and finally during supine restraint.

#### **7.2.1.1 Behaviour Recorded in the Viewing Jar.**

- The animal is placed in the viewing jar for 5 min. This is located on top of a grid which is suspended above a piece of white paper. The behaviours listed in Table 2.1 are recorded without disturbing the animal and the amount of urination or defaecation is monitored at the end of the observation period. Incidents of bizarre or stereotyped behaviour and convulsions are recorded separately.

#### **7.2.1.2 Behaviour Recorded in the Arena.**

- A metal plate is inserted under the viewing jar and the animal transferred and briskly dropped onto the centre of the floor of the arena without being handled. The stop watch is started and the immediate reaction to the new environment is recorded along with the measures detailed in Table 2.2.



#### **7.2.1.3 Behaviour Recorded on or Above the Arena.**

- After removing the animal from the arena, grip the tail between thumb and forefinger and record the observations listed in Table 2.3.

#### **7.2.1.4 Behaviour Recorded During Supine Restraint**

- Firmly scruff the animal and record the measures listed in Table 2.4. Record the final behaviours as absent, 0 or present, 1 throughout the procedure.

### **7.3 *Materials and Methods - Neurochemical Analysis***

#### **7.3.1 BACE1 KO and Tg mice**

Upon completion of behavioural testing, brains from BACE1 mice were harvested and the following regions were dissected and snap frozen and stored at -80°C until assay: dorsal striatum, nucleus accumbens, hippocampus, hypothalamus, cerebellum and frontal cortex (left and right hemispheres pooled). All reagents used were analytical grade and were purchased from Sigma Chemicals (Poole, UK). All solvents were HPLC grade and were purchased from Fisher Scientific (Loughborough, UK). Samples were probe sonicated in 0.1% w/v Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 0.01% w/v EDTA, 0.1% w/v L-cysteine, 0.4M perchloric acid (10 µl/mg tissue) and centrifuged at 10,000 rpm, 4°C for 10 minutes. Supernatant fractions were analysed using HPLC with electrochemical detection. The mobile phase consisted of 0.07M KH<sub>2</sub>PO<sub>4</sub>, 1mM octane sulphonic acid and 0.1mM Na<sub>2</sub>EDTA made up in 10% methanol, 0.5% propan-2-ol. Analytes were separated using 2 x 100 x 4.6mm i.d. C18 Speed Rod columns connected in series. Calibration curves were generated for 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT). Turnover rates were assessed by calculating the following ratios: DOPAC/DA, HVA/DA, DOPAC/HVA and 5-HIAA/5-HT.

### 7.3.2 BACE1/2 double KO mice

Brain regions were dissected from: 2 male and 2 female BACE 1/2 KO and wild type mice, snap frozen on dry ice and stored at -80°C. Brain samples were weighed and homogenised in homogenising buffer (Perchloric acid, Na metabisulphate, EDTA. Na<sub>2</sub>, cysteine, deionised water) in the ratio of 100ul homogenising buffer per mg of tissue. This resulted in a tissue concentration of 0.01g/ml. The samples were then centrifuged at 10000 x g at 4°C for 10 minutes. The supernatant was removed and stored at -80°C. 20µl of the resulting supernatant was transferred into a micro volume glass vial for HPLC-ECD (ElectroChemical Detection) analysis. HPLC Mobile Phase: 0.07M KH<sub>2</sub>PO<sub>4</sub>, 1.5mM OSA.Na, 0.1mM EDTA.Na<sub>2</sub>, 12% MeOH, 88% H<sub>2</sub>O, 0.5% THF. 10ul of each sample was injected at a flow rate of 2.5ml/min onto the columns (Chromolith Speedrod 50 x 4.6mm & 2 x 100 x 4.6mm) and compared with a previously run calibration to identify and quantify components (DOPA, DOPAC, Dopamine, 5-HIAA, 5HTP, HVA, 3-MT, 5-HT). This is measured using a Decade electrochemical detector, voltage set at +0.65 with reference.

#### 7.4 BACE1 KO SHIRPA Results

			Wild type (+/+)	Knockout (KO/KO)
	Stats test	P-Values	Median (Interquartile range)	Median (Interquartile range)
Body Position	NV	-	3 (3-3)	3 (3-3)
Spontaneous Activity	U	0.79	1 (1-1)	1 (1-1)
Respiration	NV	-	2 (2-2)	2 (2-2)
Tremor	NV	-	0 (0-0)	0 (0-0)
Transfer Arousal	U	0.48	3 (3-4)	4 (3-4)
Arena Activity	AN	0.56	14.35 ± 1.84	16.05 ± 2.16
Palprebral Closure	NV	-	0 (0-0)	0 (0-0)
Piloerection	NV	-	0 (0-0)	0 (0-0)
Startle Response	U	0.42	1.5 (1-2)	2 (1-2)
Gait	NV	-	0 (0-0)	0 (0-0)
Pelvic Elevation	U	1.00	2 (2-2)	2 (2-2)
Tail Elevation	NV	-	1 (1-1)	1 (1-1)
Touch Escape	U	0.99	2 (1-2)	2 (1-2)
Positional Passivity	NV	-	1 (1-1)	1 (1-1)
Abnormal Behaviour	F	0.50	0 (0-0)	0 (0-0)
Visual Placing	U	0.79	3 (3-3)	3 (2.5-3)
Grip Strength	U	0.73	2 (2-2)	2 (2-3)
Body Tone	NV	-	1 (1-1)	1 (1-1)
Toe Pinch	U	0.27	3 (2-3)	3 (2.5-3)
Wire Manoeuvre	U	<b>0.007</b>	<b>2 (1-2)</b>	<b>0.5 (0-1)</b>
Pinna	NV	-	1 (1-1)	1 (1-1)
Cornea	NV	-	1 (1-1)	1 (1-1)
Heart Rate	NV	-	1 (1-1)	1 (1-1)
Limb Tone	U	<b>0.05</b>	<b>2 (2-2)</b>	<b>2 (1-2)</b>
Abdominal Tone	NV	-	1 (1-1)	1 (1-1)
Lacrimation	NV	-	0 (0-0)	0 (0-0)
Salivation	F	0.50	1 (1-1)	1 (1-1)
Provoked Biting	U	1.00	0 (0-0)	0 (0-0)
Right Reflex	NV	-	0 (0-0)	0 (0-0)
Contact Righting	NV	-	1 (1-1)	1 (1-1)
Negative Geotaxis	U	0.63	0 (0-0)	0 (0-0.5)
Fear	NV	-	0 (0-0)	0 (0-0)
Irritability	U	0.42	0 (0-1)	0 (0-0)
Aggression	F	0.50	0 (0-0)	0 (0-0)
Vocalisation	U	0.28	1 (0-1)	0 (0-1)
Faeces	AN	<b>0.02</b>	<b>3.95 ± 0.51</b>	<b>5.90 ± 0.63</b>
Urination	AN	0.33	0.55 ± 0.15	0.75 ± 0.16
Body Weight	AN	0.45	28.25 ± 0.93	27.42 ± 1.27
Body Length	AN	0.31	96.65 ± 1.19	95.20 ± 1.45

Summary of Primary Screening Results Grouped by genotype. Data are shown as median with quartile range in parentheses except where data are expressed as mean ± SEM. Significant measures are in bold type. AN = ANOVA, U = Mann Whitney U test, F = Fishers Exact Test, NV = No Variability.

## 7.5 BACE1 Line 17 SHIRPA Results

			Wild type (+/+)	Transgenic (Tg/+)
	Stats test	P-Values	Median (Interquartile range)	Median (Interquartile range)
Body Position	NV	-	3 (3-3)	3 (3-3)
Spontaneous Activity	U	0.79	1 (1-1)	1 (1-1)
Respiration	NV	-	2 (2-2)	2 (2-2)
Tremor	NV	-	0 (0-0)	0 (0-0)
Transfer Arousal	U	0.68	3 (3-4)	3 (3-4)
Arena Activity	AN	0.95	24.4 ± 1.13	24.5 ± 0.99
Palprebral Closure	NV	-	0 (0-0)	0 (0-0)
Piloerection	NV	-	0 (0-0)	0 (0-0)
Startle Response	U	0.59	2 (2-2)	2 (2-2)
Gait	NV	-	0 (0-0)	0 (0-0)
Pelvic Elevation	NV	-	2 (2-2)	2 (2-2)
Tail Elevation	U	1.00	1 (1-1)	1 (1-1)
Touch Escape	U	0.62	2 (2-2)	2 (2-2)
Positional Passivity	NV	-	1 (1-1)	1 (1-1)
Abnormal Behaviour	NV	-	0 (0-0)	0 (0-0)
Visual Placing	F	0.24	3 (3-3)	3 (3-3)
Grip Strength	U	0.84	2 (2-3)	2 (2-2.5)
Body Tone	NV	-	1 (1-1)	1 (1-1)
Toe Pinch	U	0.79	3 (3-3)	3 (3-3)
Wire Manoeuvre	U	0.30	0 (0-1)	0 (0-0.5)
Pinna	NV	-	1 (1-1)	1 (1-1)
Cornea	NV	-	1 (1-1)	1 (1-1)
Heart Rate	NV	-	1 (1-1)	1 (1-1)
Limb Tone	U	0.82	2 (2-2)	2 (2-2)
Abdominal Tone	NV	-	1 (1-1)	1 (1-1)
Lacrimation	NV	-	0 (0-0)	0 (0-0)
Salivation	NV	-	1 (1-1)	1 (1-1)
Provoked Biting	U	0.28	0 (0-0.5)	0 (0-1)
Right Reflex	NV	-	0 (0-0)	0 (0-0)
Contact Righting	NV	-	1 (1-1)	1 (1-1)
Negative Geotaxis	F	0.50	0 (0-0)	0 (0-0)
Fear	NV	-	0 (0-0)	0 (0-0)
Irritability	U	0.42	0 (0-1)	0 (0-1)
Aggression	NV	-	0 (0-0)	0 (0-0)
Vocalisation	U	1.00	1 (0-1)	1 (0-1)
Faeces	AN	0.42	3.05 ± 0.41	3.45 ± 0.27
Urination	AN	0.55	0.25 ± 0.10	0.35 ± 0.27
Body Weight	AN	0.46	22.46 ± 0.55	22.13 ± 0.54
Body Length	AN	0.22	84.60 ± 0.94	83.25 ± 0.97

Summary of Primary Screening Results Grouped by genotype. Data are shown as median with quartile range in parentheses except where data are expressed as mean ± SEM. Significant measures are in bold type. AN = ANOVA, U = Mann Whitney U test, F = Fishers Exact Test, NV = No Variability.

## 7.6 BACE1 Line 4 Tg SHIRPA Results

			Wild type (+/+)	Transgenic (Tg/+)
	Stats test	P-Values	Median (Interquartile range)	Median (Interquartile range)
Body Position	NV	-	3 (3-3)	3 (3-3)
Spontaneous Activity	U	0.79	1 (1-1)	1 (1-1)
Respiration	NV	-	2 (2-2)	2 (2-2)
Tremor	NV	-	0 (0-0)	0 (0-0)
Transfer Arousal	U	0.13	4 (3-4)	3 (3-4)
<b>Arena Activity</b>	<b>AN</b>	<b>0.002</b>	<b>22.95 ± 0.96</b>	<b>17.90 ± 1.15</b>
Palprebral Closure	NV	-	0 (0-0)	0 (0-0)
Piloerection	NV	-	0 (0-0)	0 (0-0)
Startle Response	U	0.70	2 (2-2)	2 (1.5-2)
Gait	F	0.50	0 (0-0)	0 (0-0)
Pelvic Elevation	U	0.79	2 (2-2)	2 (2-2)
Tail Elevation	F	0.24	1 (1-1)	1 (1-1)
Touch Escape	U	0.42	2 (2-3)	2 (2-2.5)
Positional Passivity	NV	-	1 (1-1)	1 (1-1)
Abnormal Behaviour	NV	-	0 (0-0)	0 (0-0)
Visual Placing	U	0.42	3 (2.5-3)	3 (3-3)
Grip Strength	U	0.31	2 (2-3)	2 (2-2)
Body Tone	NV	-	1 (1-1)	1 (1-1)
Toe Pinch	U	0.87	3 (2-3)	3 (2-3)
Wire Manoeuvre	U	0.29	0 (0-0)	0 (0-1)
Pinna	NV	-	1 (1-1)	1 (1-1)
Cornea	NV	-	1 (1-1)	1 (1-1)
Heart Rate	F	0.50	1 (1-1)	1 (1-1)
Limb Tone	U	1.00	2 (2-2)	2 (2-2)
Abdominal Tone	F	0.50	1 (1-1)	1 (1-1)
Lacrimation	NV	-	0 (0-0)	0 (0-0)
Salivation	NV	-	1 (1-1)	1 (1-1)
<b>Provoked Biting</b>	<b>U</b>	<b>0.06</b>	<b>0 (0-0.5)</b>	<b>1 (0-1)</b>
Right Reflex	NV	-	0 (0-0)	0 (0-0)
Contact Righting	NV	-	1 (1-1)	1 (1-1)
Negative Geotaxis	F	0.50	0 (0-0)	0 (0-0)
Fear	NV	-	0 (0-0)	0 (0-0)
Irritability	U	0.59	0 (0-1)	0 (0-1)
Aggression	U	1.00	0 (0-0)	0 (0-0)
Vocalisation	U	0.42	1 (0-1)	1 (0.5-1)
Faeces	AN	0.42	3.65 ± 0.32	3.20 ± 0.44
Urination	AN	0.18	0.10 ± 0.07	0.30 ± 0.13
Body Weight	AN	0.02	22.17 ± 0.58	21.15 ± 0.63
Body Length	AN	0.11	85.75 ± 0.92	84.15 ± 0.82

Summary of Primary Screening Results Grouped by genotype. Data are shown as median with quartile range in parentheses except where data are expressed as mean ± SEM. Significant measures are in bold type. AN = ANOVA, U = Mann Whitney U test, F = Fishers Exact Test, NV = No Variability.

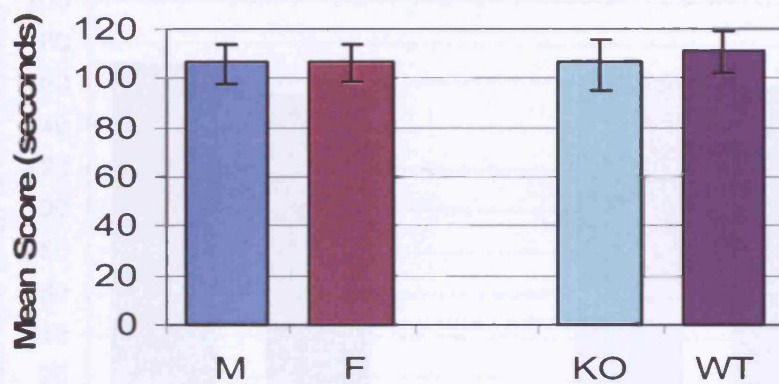


## 7.7 BACTAS dTg SHIRPA Results

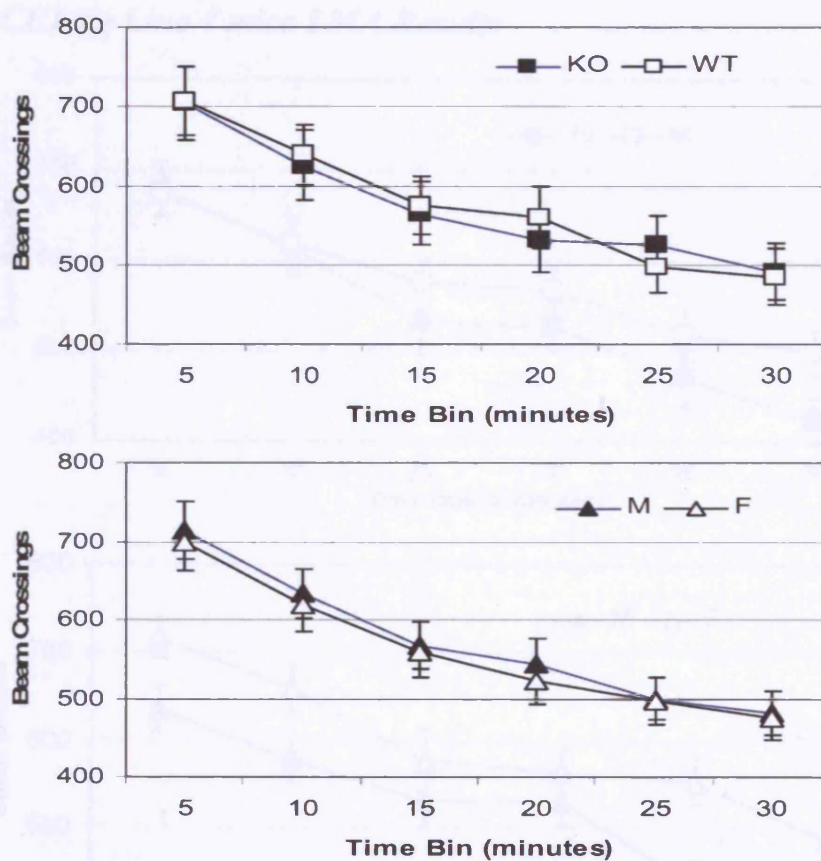
			Wild type (+/+)	Transgenic (dTg/-)
	Stats test	P-Values	Median (Interquartile range)	Median (Interquartile range)
Body Position	U	0.86	3 (3-3)	3 (3-3)
Spontaneous Activity	U	1.00	1 (1-1)	1 (1-1)
Respiration	NV	-	2 (2-2)	2 (2-2)
<b>Tremor</b>	U	<b>0.001</b>	<b>0 (0-0.5)</b>	<b>1 (1-1)</b>
Transfer Arousal	U	0.34	3 (2-3)	3 (2-3)
<b>Arena Activity</b>	AN	<b>0.036</b>	<b>5.1 ± 0.8</b>	<b>8.3 ± 1.4</b>
Palprebral Closure	NV	-	0 (0-0)	0 (0-0)
Piloerection	U	0.17	0 (0-1)	1 (0-1)
Gait	U	0.65	0 (0-0)	0 (0-1)
Pelvic Elevation	U	0.76	2 (2-2)	2 (2-2)
Tail Elevation	U	1.00	1 (1-1)	1 (1-1)
Touch Escape	U	0.40	2 (2-2)	2 (1-2)
Positional Passivity	F	0.17	1 (1-1)	1 (1-1)
Abnormal Behaviour	NV	-	0 (0-0)	0 (0-0)
Visual Placing	NV	-	3 (3-3)	3 (3-3)
Grip Strength	U	0.76	2 (2-2)	2 (2-2)
Body Tone	U	0.83	1 (0-1)	1 (0-1)
Wire Manoeuvre	U	0.15	3 (3-3.5)	3 (2-3)
Heart Rate	NV	-	1 (1-1)	1 (1-1)
Limb Tone	U	0.09	1 (1-1)	1 (1-1)
Abdominal Tone	F	0.07	1 (1-1)	1 (1-1)
Lacrimation	NV	-	0 (0-0)	0 (0-0)
Salivation	NV	-	0 (0-0)	0 (0-0)
<b>Provoked Biting</b>	F	<b>&lt;0.001</b>	<b>1 (1-1)</b>	<b>0 (0-1)</b>
Right Reflex	NV	-	0 (0-0)	0 (0-0)
Contact Righting	NV	-	1 (1-1)	1 (1-1)
Negative Geotaxis	U	1.00	1 (0-2.5)	4 (0-4)
Fear	U	0.83	0 (0-0)	0 (0-0)
Irritability	NV	-	0 (0-0)	0 (0-0)
<b>Aggression</b>	AN	<b>0.002</b>	<b>0 (0-0)</b>	<b>0 (0-1)</b>
Vocalisation	NV	-	0 (0-0)	0 (0-0)
Faeces	AN	0.21	1.7 ± 0.4	2.6 ± 0.5
Urination	AN	0.41	0.1 ± 0.1	0.0 ± 0.0
<b>Body Weight (g)</b>	AN	<b>0.001</b>	<b>40.6 ± 1.3</b>	<b>34.3 ± 1.6</b>
<b>Body Length (mm)</b>	AN	<b>0.001</b>	<b>97.0 ± 0.9</b>	<b>93.7 ± 1.0</b>

Summary of Primary Screening Results Grouped by genotype. Data are shown as median with quartile range in parentheses except where data are expressed as mean ± SEM. Significant measures are in bold type. AN = ANOVA, U = Mann Whitney U test, F = Fishers Exact Test, NV = No Variability.

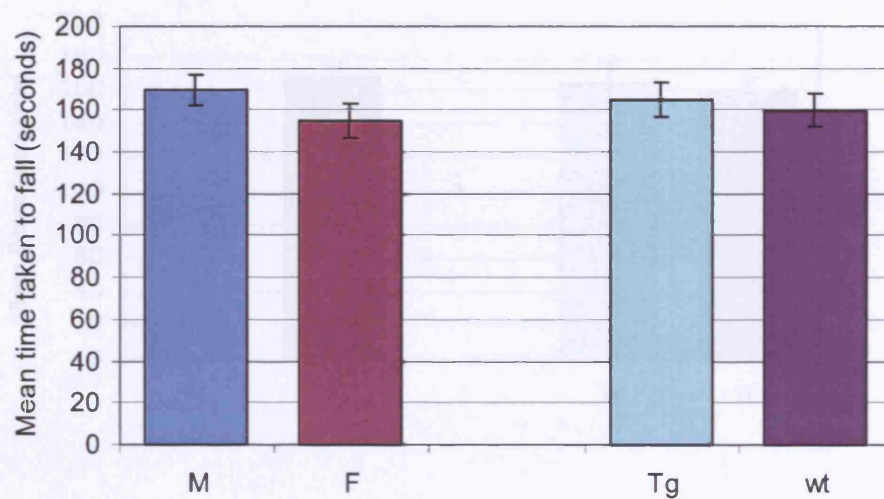
### 7.8 *BACE1* KO mice Rotarod Results



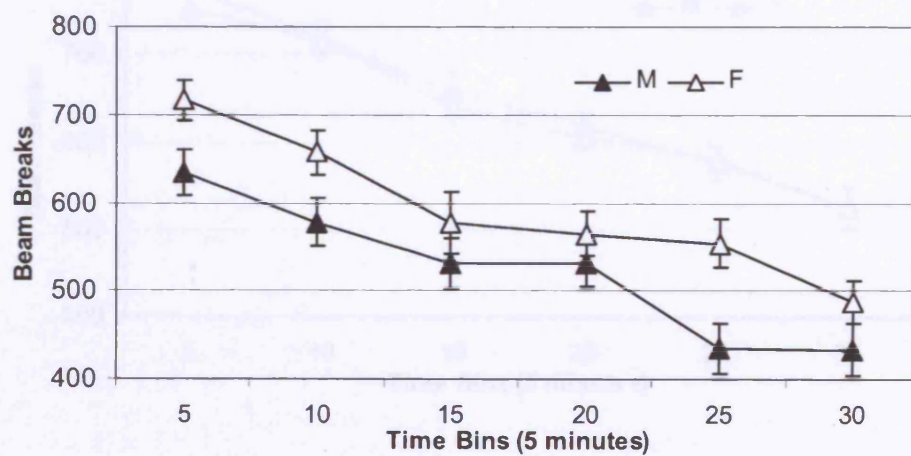
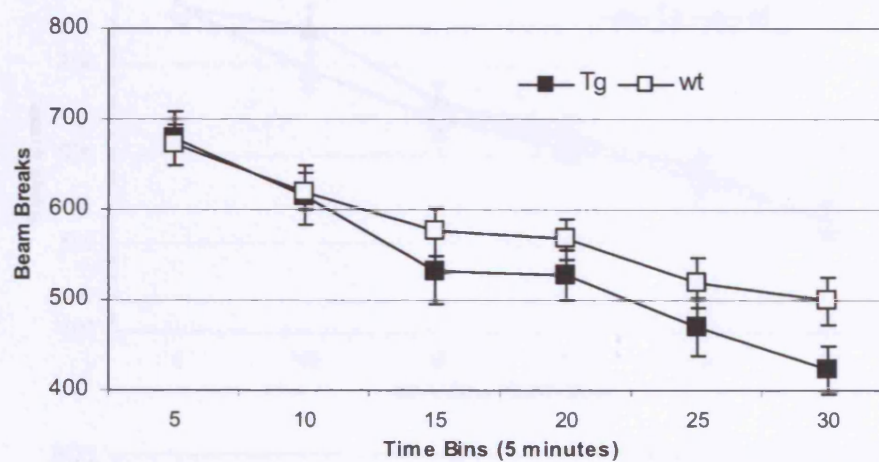
### 7.9 *BACE1* KO mice LMA Results



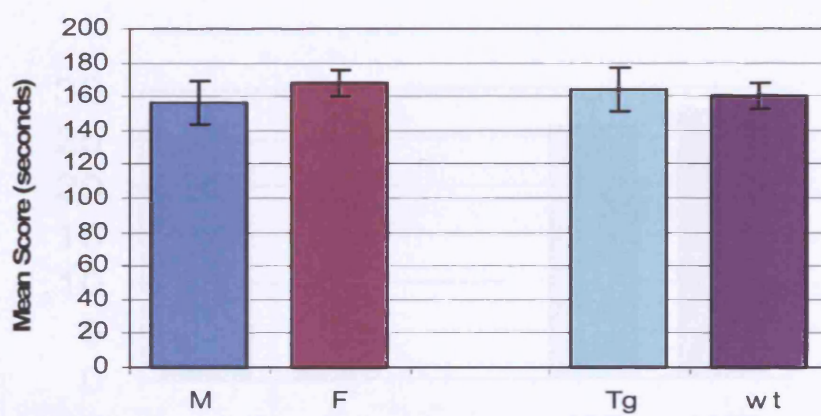
### 7.10 BACE1 Tg Line 4 mice Rotarod Results



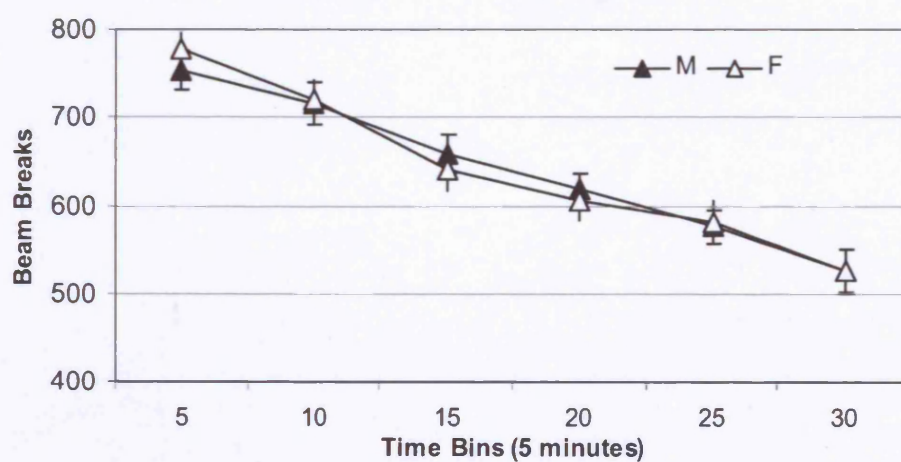
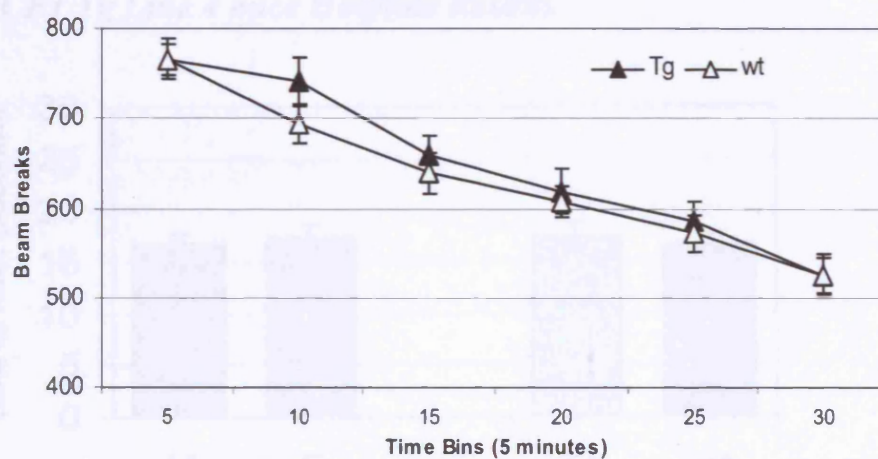
### 7.11 BACE1 Tg Line 4 mice LMA Results



### 7.12 BACE1 Tg Line 17 mice Rotarod Results

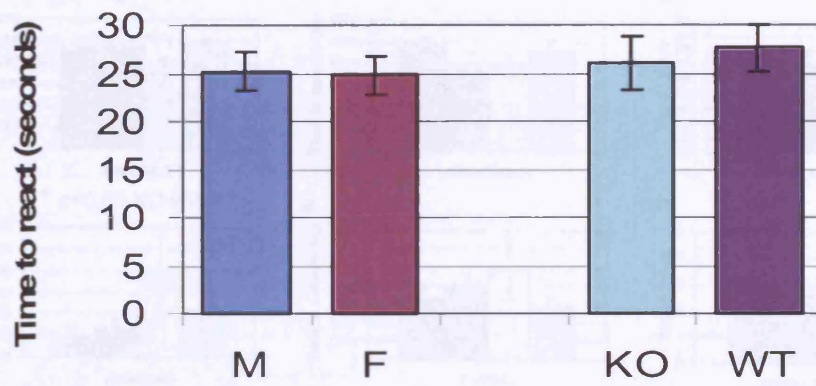


### 7.13 BACE1 Tg Line 17 mice LMA Results

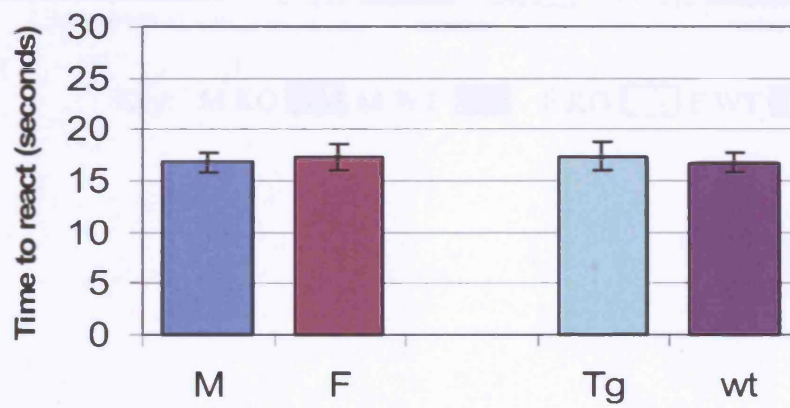




#### 7.14 *BACE1 KO mice Hotplate Results*

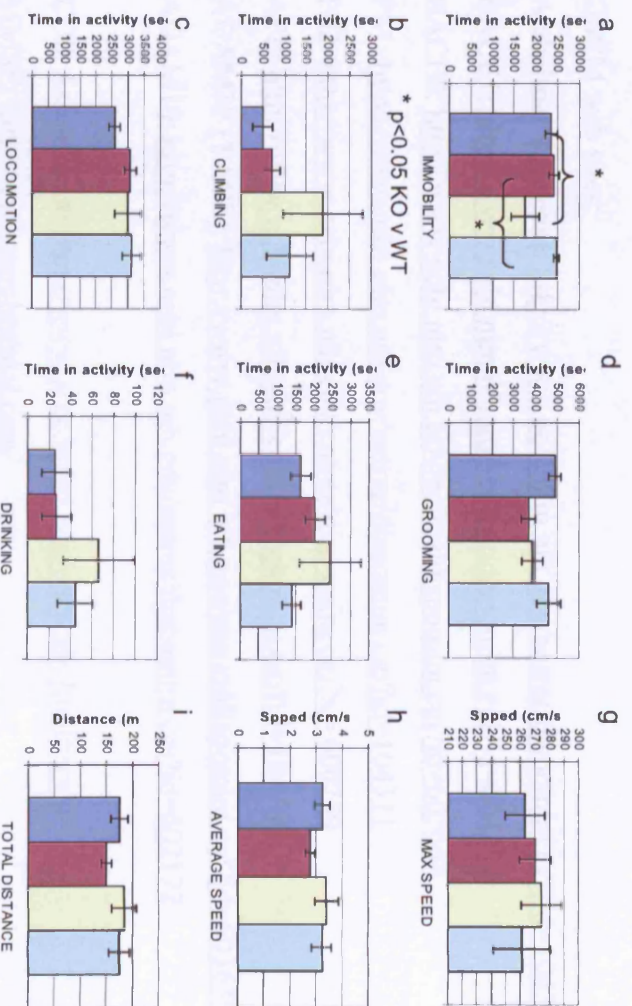


#### 7.15 *BACE1 Tg Line 4 mice Hotplate Results*





## 7.16 BACE1/2 double KO LABORASTM Results



Key: M KO M WT F KO F WT F WT

### 7.17 On line data sources

OMIM web sites:

Alzheimer's Disease: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=104300>

BACE1: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=604252>

BACE2: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=605668>

PS1: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=104311>

PS2: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=600759>

APP: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=104760>

ADAM17 (TACE): <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=603639>

ADAM10: <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=602192>

CDR Assessment: <http://www.adrc.wustl.edu/cdrScale.html#scale>

MMSE: <http://www.minimental.com/>

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